Inhibin, Activin and Follistatin in Human Reproductive Physiology

Editors:
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and
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Imperial College Press

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FOREWORD

The story of the inhibin represents one of the major advances in our knowledge of reproductive endocrinology in the past two decades. It represents an excellent example of the power of clinical observation and investigation. The original description of the pituitary cytological changes that followed testicular irradiation, and their reversal by aqueous testicular extracts, remained relatively unexplored until the advent of sensitive radioimmunoassays for follicle-stimulating hormone (FSH) and their application to the study of the endocrinology of male infertility. Several clinical investigators from all over the world more or less simultaneously published their findings, that many men with seminiferous tubule failure, leading to infertility, had elevated serum FSH levels, while luteinising hormone (LH) and testosterone often remained within the normal range for fertile controls. This gave rise to the hypothesis that spermatogenic failure might lead to diminished secretion of a seminiferous epithelium-related feedback factor, corresponding to the activity originally found in testicular extracts. Deficiency of a possibly related factor was postulated to account for the selectively elevated FSH levels observed in women as menopause approached. Initial efforts to isolate the active principle from testicular extracts proved to be fruitless, despite confirmation of the ability of both testicular extracts and ovarian follicular fluid to induce selective FSH suppression when administered in several animal models. The demonstration by the late Paul Franchimont that seminal plasma from normal men lowered FSH levels in castrate male rats, while seminal plasma from men with severe seminiferous tubule failure did not, was highly significant as a very early stimulus to the quest for inhibin.

The initial successful isolation and purification of inhibin were achieved, using bovine follicular fluid as the source, by a group working in Melbourne, Australia. This was followed rapidly by success in several other laboratories. The Melbourne effort had taken almost 15 years and was ultimately the result of meticulous protein chemistry and the use of a robust

bioassay, in which non-specific inhibitory effects of FSH secretion were carefully monitored.

With the remarkable rapidity which characterises modern molecular techniques, the cloning of the inhibin was quickly achieved and its structural properties determined. It was recognised that they belonged to the family of transforming growth factors. Unexpectedly, in the course of efforts at inhibin purification, fractions causing FSH stimulation were isolated by two groups simultaneously, and found to be dimers of the inhibin subunit. They were christened "activins". An additional unrelated FSH inhibitory peptide termed FSH-suppressing protein or follistatin, was isolated by two independent groups and found to be an activin-binding agent. With the establishment of sensitive immunoassays for the inhibin, activin and follistatin, progress in elucidating their physiology was again rapid. The publication of the present book provides a summary of the current state of knowledge by an international group of workers in this field, particularly those based in the UK, and is timely and comprehensive.

Despite the enormous progress in this field, and despite the information covered in this book, the "messages" regarding the inhibin and activin are not yet widely appreciated. The present work will surely help to correct this state of affairs. Future research should help to define the physiology of the inhibin, including isolation of the receptor, the significance of the relatively large amounts of subunit which circulate, the diagnostic roles of the inhibin (particularly in pregnancy and in ovarian cancer), and the question of whether they may have contraceptive potential — a hope that was an early stimulus to the funding of inhibin researchers.

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CHAPTER 1

INTRODUCTION

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Reproduction in mammals is controlled by complex systems involving multiple neural, neuroendocrine, endocrine and paracrine cell–cell communication pathways. The roles of hypothalamic gonadotrophin releasing hormone (GnRH), pituitary gonadotrophins (follicle stimulating hormone, FSH and luteinising hormone, LH) and gonadal steroids are well established but there is increasing evidence for the involvement of various cytokines and growth factors at each level of the hypothalamic-pituitary-gonadal (H-P-O) axis. These include inhibin and activin, dimeric glycoproteins belonging to the TGF-β superfamily, and follistatin a structurally distinct though functionally related molecule. The biological significance of these proteins in reproduction, their sites of expression, physiological actions, mechanisms of action and methods for their detection and measurement are amongst the topics covered in the accompanying chapters of this book. This introductory chapter presents a brief historical account of the discovery and characterisation of inhibin, activin and follistatin and touches upon their main biological functions.

1.1 Historical Background

The concept that the gonads produce a non-steroidal regulator of anterior pituitary gland function developed more than 75 years ago with the observation that destruction of seminiferous tubules in rats by X-irradiation of the testis caused hypertrophy of the anterior pituitary gland (Motram & Cramer 1923). A decade later it was demonstrated that a water soluble extract from bull testis could prevent this post-castration hypertrophy of the pituitary in rats (McCullagh 1932). The active substance responsible for this action was named "inhibin". The quest for inhibin did not advance further until three decades later when the pituitary gonadotrophins FSH and LH were isolated and characterised in the 1960s (Sairam & Papkoff 1974) and radio-immunoassays were developed to quantify these hormones in tissues and body fluids (Midgely 1966, 1967). The ability to measure serum FSH and LH with relative ease revived interest in the inhibin concept, which offered an explanation for the differential regulation of FSH and LH release often observed in humans and animals but not accounted for by known interactions between gonadal steroids and GnRH at the hypothalamic or pituitary level (reviewed by Franchimont *et al.* 1979a, 1979b and Setchell *et al.* 1977).

Sherman & Koreman (1975) first postulated the presence of inhibin-like activity (ILA) in ovarian follicular fluid (FF) and direct evidence to support this was provided by de Jong & Sharpe (1976) who observed that administration of steroid free bovine follicular fluid (bFF) to castrated rats suppressed serum FSH levels. Thus, the inhibin concept, which arose from studies on male animals, was extended to females. With the aid of an improved bioassay for inhibin, based on the suppression of FSH production by rat pituitary cells *in vitro*, several research groups eventually succeeded in isolating and characterising inhibin from ovarian follicular fluid in the mid 1980s (Ling *et al.* 1985, Miyamato *et al.* 1985, Rivier *et al.* 1985, Robertson *et al.* 1985, 1986, Knight *et al.* 1987). Cloning of the genes encoding inhibin soon followed (Mason *et al.* 1985, Forage *et al.* 1986, Woodruff *et al.* 1987), allowing recombinant DNA-derived inhibin to be produced (Mason *et al.* 1987, Tierney *et al.* 1990). Although it has taken more than five decades to

prove the validity of the inhibin concept, the pace of progress has accelerated dramatically in the last decade. There have been numerous unexpected twists and turns along the way, not least of which was the discovery of activin and follistatin. Like inhibin, these molecules were first identified through their ability to modulate pituitary FSH secretion but their actions extend far beyond this.

1.2 Characterisation of Inhibin

Most investigators reported an apparent molecular weight of ~32 kDa for the biologically active inhibin protein although a higher molecular weight form (58 kDa) was initially isolated from bFF (Robertson *et al.* 1985). SDS-PAGE analysis revealed that the 32-kDa inhibin was a heterodimer composed of an α-subunit (20 kDa) and a β-subunit (~14 kDa) linked by disulphide bonds (Ling *et al.* 1985, Fukuda *et al.* 1986, Robertson *et al.* 1986). These subunits are encoded by different genes and represent the carboxy terminal regions of larger precursor molecules that undergo post-translational processing after synthesis. The 58-kDa form has an amino terminally extended α-subunit (44 kDa) with an identical β-subunit (14 kDa). Two isoforms of 32-kDa inhibin (inhibins A and B) were isolated from porcine follicular fluid (pFF); these share the same α-subunit but have slightly different β subunits, that are encoded by different genes, referred to as β_A and β_B respectively (Ling *et al.* 1985). Inhibins A and B were found to have similar bioactivity (Ling *et al.* 1985). Immunoblotting techniques have identified different forms of inhibin-αβ dimer in bFF with Mw values of 120, 108, 88, 65, 55 and 32 kDa. However, it is clear that the 32-kDa inhibin is the mature fully processed form of inhibin and is probably the major dimeric form present in the peripheral circulation. It should be noted that several forms of inhibin α-subunit monomer have also been identified in and isolated from FF (Knight 1991). While these "free" inhibin α-subunit forms lack classical inhibin-like biological activity, they are present in considerable amounts in the peripheral circulation and complicate the interpretation of results from early inhibin radioimmunoassays developed in the mid-late 1980s.

Human, porcine, bovine and rat inhibins are closely related in structure and highly conserved. Comparison of the mature α subunits from different species indicating 85% homology at the amino acid level. While the mature β_A-subunit of these four species are identical, the β_B subunits differ in one amino acid between human and porcine inhibins and three amino acid differences between human and rat β_B subunits. Homology between β_A and β_B subunits is approximately 80%.

A high degree of sequence conservation has also been observed in the precursor regions of the α and β subunits, implicating the existence of potentially important biologically active peptides within these amino terminal regions. The almost identical exon-intron structure of the genes for α and β subunits further indicates that inhibin is highly conserved between species (Ying 1988).

1.3 Activin

The purification of inhibin from pFF led to the identification of activin in side fractions obtained during chromatographic procedures (Vale *et al.* 1986, Ling *et al.* 1986). Activin was identified and isolated owing to their ability to increase FSH release from pituitary gonadotrophs *in vitro*. Multiple forms of activin exist, each being a dimer of two mature inhibin β subunits and having a molecular weight of ~25 kDa. Activin-A is a homodimer consisting of two β_A subunits and activin-B is a homodimer made up of two β_B subunits; activin-AB is a heterodimer consisting of a β_A-subunit linked to a β_B-subunit. Activin-A is identical to the erythroid differentiation factor which was isolated independently from a human leukaemic cell line (Eto *et al.* 1987). Further information on the tissue origin, mechanism of action, quantitation methods and functional role of activin in relation to human reproduction will be found elsewhere in this book.

Recently additional isoforms of inhibin/activin β-subunit, termed β_C, β_D and β_E, have been cloned from human liver (Hotten *et al.* 1995), *Xenopus laevis* (Oda *et al.* 1995) and mouse liver (Fang *et al.* 1996), respectively. Expression of β_C mRNA has been reported in human ovary, placenta

and testis (Loveland *et al.* 1996), suggesting possible reproductive roles. β_C-subunit has 53% and 51% of homology to mature β_A and β_B subunits respectively. There is 63% similarity between β_C and β_D subunits. The predicted mature region of β_E-subunit has >60% homology to the amino acid sequence of β_C and β_D subunits and ~45% identity to β_A and β_B subunits. The existence of these three additional subunits raises the possibility of the existence of activins C, D and E and potentially nine heterodimeric activin forms. There is also the potential for three more molecular isoforms of inhibin-$\alpha\beta$ dimer. It remains to be established which, if any, of these homo- and heterodimers are actually synthesised naturally and their potential biological roles remain obscure. The synthesis of recombinant activin-C and activin-E has recently been reported (Kron *et al.* 1998).

1.4 Follistatin

Follistatin is a single chain glycosylated polypeptide that was also identified in chromatographic side fractions generated during the purification of inhibin from follicular fluid (Ying 1988). Follistatin bears no structural similarity to inhibin but it too was found to suppress FSH release in pituitary cell inhibin bioassays, albeit with a potency only 10–30% that of inhibin. Although the molecule is encoded by a single gene, alternative mRNA splicing giving rise to two different precursor forms: follistatin (FS) 344 and precursor FS317 (Shimasaki 1988, Fukui *et al.* 1993). Post-translational processing of both FS315 and FS288 yields up to six molecular forms of follistatin which vary in their truncation of the carboxyl terminal and the presence of carbohydrate chains (Sugino *et al.* 1993).

Follistatin has been identified as a high-affinity activin-binding protein which modulates the biological availability of activin at the target cell level (Nakamura *et al.* 1990). While follistatin binds to both activin and inhibin via their common β-subunit (Shimonaka *et al.* 1991), evidence suggests that it is only capable of neutralising the biological activity of activin. Follistatin has also been shown to have a high affinity for heparin sulphate chains of cell surface proteoglycans (Nakamura *et al.* 1991). The shortest form FS288

has the highest affinity, and the longest form FS315 has the lowest affinity (Sugino *et al.* 1993). Further details of the structure, source, methods of quantification and biological functions of follistatin may be found in Chapters 2 and 6 of this book.

1.5 Biological Functions

Much progress has been made in the last decade towards identifying physiological and pathophysiological roles of inhibin, activin and follistatin in the reproductive system but the picture is far from complete, certainly with regard to human. Progress has accelerated in recent years, in part due to: (i) development of improved methods for detecting and quantifying these proteins, (ii) availability of recombinant DNA-derived inhibin, activin and follistatin, (iii) development of improved *in vitro* model systems, (iv) development of "gene knockout" transgenic animal models, and (v) recognition of the potential utility of these molecules in clinical diagnosis. Although all three molecules were first isolated from gonadal fluids on the basis of their ability to modulate pituitary FSH secretion, they are now known to have a much broader spectrum of actions both within and outside the reproductive system. In fact, while a classical negative feedback action of gonadal inhibin on pituitary FSH secretion is well established, there is very little evidence to support feedback actions of gonadally-derived activin or follistatin on gonadotrophin secretion. In terms of their reproductive roles, these are most likely confined to local autocrine/paracrine actions (i.e. at the level of the ovary, testis, prostate, anterior pituitary and placenta) and involve modulation of cell proliferation/differentiation. Expression of inhibin/activin subunit mRNAs and follistatin mRNA occurs in many extragonadal tissues and there is compelling evidence, at least in rats, that activin and follistatin synthesised within the anterior pituitary contribute to the regulation of gonadotrophin secretion by modulating gonadotroph responsiveness to GnRH, gonadal steroids and inhibin (DePaolo 1997). Inhibin α-subunit has been implicated as a tumour suppressor protein (Matzuk *et al.* 1992) while activin has been shown to act in a number of reproductive and "non-reproductive" tissues to promote cell proliferation and differentiation. Functions identified

for activin in "non-reproductive" tissues include erythropoiesis, wound repair, bone morphogenesis and mesoderm formation (reviewed by Mather *et al.* 1997). In many of the above examples, follistatin has been shown to modulate the effects of activin, consistent with its properties as an activin-binding protein. Further detailed coverage of the reproductive functions of inhibin, activin and follistatin is presented in the following chapters.

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CHAPTER 2

DEVELOPMENT OF IMMUNOASSAYS FOR INHIBIN, ACTIVIN AND FOLLISTATIN

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Inhibin, activin and follistatin biology research expanded rapidly in the 1980s when these molecules were purified (Ling *et al.* 1985, Miyamoto *et al.* 1985, Rivier *et al.* 1985, Robertson *et al.* 1985, Ling *et al.* 1986a, 1986b, Vale *et al.* 1986, Ueno *et al.* 1987) and had their genes cloned (Mason *et al.* 1986, Esch *et al.* 1987, Shimasaki *et al.* 1988). However, bioassays proved to possess inadequate specificity to measure these functional entities reliably in complex fluids. The literature shows that early inhibin and activin immunoassays cross-reacted with many different forms, some of which had no biological function (Robertson 1990). By the early 1990s progress slowed down when these limitations became apparent. In response to the obvious need, our laboratory has produced a family of specific and ultrasensitive enzyme-immunoassays for inhibin-A, inhibin-B, pro-α_C, activin-A, activin-AB and follistatin. These have now been widely applied to a variety of research areas, some of which are illustrated in the other chapters of this book. In this chapter, we review the history of inhibin, activin and follistatin assays. We then describe the development of the new enzyme-immunoassays from our laboratory and their advantages and disadvantages. Finally we give an opinion about further assays which might be desirable for other inhibin, activin and follistatin forms.

2.1 Structures of Inhibin, Activin and Follistatin

Both inhibin and activin are members of the structurally similar but functionally diverse transforming growth factor-β superfamily (Mason *et al.* 1985). Inhibin was initially isolated and characterised by its ability to inhibit the production and secretion of follicle stimulating hormone (FSH) from the pituitary gland (Burger & Igarashi 1988). The mature bioactive inhibin molecule consists of an α-subunit (Mw ~20 kD) linked by disulphide bonds to one of two highly homologous β subunits (Mw ~14 kD) to form either inhibin-A [$\alpha\beta_A$] or inhibin-B [$\alpha\beta_B$]. Each subunit is initially assembled as a large pre-pro-protein which possesses an N-terminal signal peptide which targets the polypeptide to the endoplasmic reticulum, a pro-region which is needed for the accurate folding and dimerisation of the subunits, and the mature C-terminal domain. The pro-region is partially cleaved from the inhibin and activin proteins prior to their secretion resulting in the bioactive forms (Gray & Mason 1990). Evidence has been found for the existence of at least nine biologically active forms of inhibin ranging in molecular weights from 32 to >100 kD in both bovine and human plasma (Robertson *et al.* 1995, Robertson *et al.* 1996, Good *et al.* 1995). These forms arise as a result of the partial processing of high molecular weight pro-forms giving rise to amino-terminally extended α and/or β subunits, and as a result of the extent of glycosylation on the C-terminus of the α-subunit (Miyamato *et al.* 1986, Robertson *et al.* 1985, Sugino *et al.* 1992). Non-bioactive forms of the α-subunit have also been found to exist, both as pro-α_N-α_C and the further processed form pro-α_C. Despite its lack of activity in the pituitary FSH-release bioassay for inhibin, it is not yet clear whether these derivatives have any biological effect on the reproductive system. However, there is some evidence to suggest that the inhibin α-subunit may be the major form of inhibin present in certain tissues and reproductive tract tumours (Russell & Findlay 1995).

Activin exists as homodimers or heterodimers of the inhibin β subunits forming activin-A [$\beta_A\beta_A$], activin-B [$\beta_B\beta_B$] and activin-AB [$\beta_A\beta_B$]. The β_A-subunit is 100% conserved between cow, sheep, pig and human, whereas the β_B-subunit found in human, cow, sheep, pig and rat shows ~95%

conservation. These mature dimers were characterised by their ability to stimulate the production of FSH from the pituitary (Vale *et al.* 1986) and exist with molecular weights of ~24 kD (Ling *et al.* 1986a). However, several exceptions have been found whereby the subunit contains either the whole or partially processed pro-region even after secretion (Robertson *et al.* 1985, Robertson *et al.* 1986, Robertson *et al.* 1995) resulting in higher molecular weight dimers with lower bioactivity. β_A-subunit monomers have also been detected in bovine follicular fluid and are less active (18–45%) than the dimer (Robertson *et al.* 1992). Recent cloning experiments have provided evidence for the possible existence of three further β subunits. Activin β_C-subunit was cloned from a human liver cDNA library and possessed 53% and 51% homology to the amino acid sequence of the mature β_A and β_B subunits (Hötten *et al.* 1995). An activin β_D-subunit cDNA has been cloned from a *Xenopus laevis* cDNA library and showed 63% identity to the β_C protein (Oda *et al.* 1995), and most recently an activin β_E-subunit has been cloned from a mouse liver cDNA library. The predicted mature region of this subunit showed >60% homology to the amino acid sequences of activin β_C and β_D subunits, and approximately 45% homology to activin β_A and β_B subunits (Fang *et al.* 1996). Although the presence of these new subunits was originally thought to be exclusively in the liver, Loveland *et al.* (1996) detected the expression of β_C-subunit mRNA in human ovary, placenta and testis samples, and also in rat testis and spleen. To date, only purified recombinant activin-C and recombinant activin-E have been produced (Kron *et al.* 1998). The bioactivity of these two proteins has yet to be reported, although the activin-β_D has been shown to possess activin-like bioactivity in *Xenopus* embryos (Oda *et al.* 1995). The existence of these recently cloned β subunits raises the issue of the possible existence of five activin homodimers and potentially ten heterodimers. There is also the potential for five inhibins, although recent work raises questions as to whether the new activin subunits will pair with the α-subunit.

Follistatin (FS) is a cysteine-rich, single chain, glycosylated polypeptide of which three forms (31, 35 and 39 kD) were originally identified in bFF (bovine follicular fluid), with two forms found in porcine FF (Robertson *et al.* 1987, Shimasaki *et al.* 1988). Follistatin was initially purified from

pFF (Ying *et al.* 1987, Ueno *et al.* 1987) and bovine FF (Robertson *et al.* 1987) based on its ability to suppress pituitary FSH release from cultured rat anterior pituitary cells. However, the potency of follistatin in this regard was found to be 10–30% that of inhibin both *in vivo* (de Paolo *et al.* 1991) and *in vitro* (Ying *et al.* 1987, Robertson *et al.* 1987, Robertson *et al.* 1990, Wang *et al.* 1990). Thus, follistatin was thought to be a weak agonist of inhibin. However, whilst searching for activin receptors in rat ovaries Nakamura *et al.* (1990) made the important discovery that follistatin could bind to activin. Subsequent studies have also found this to be the case with follistatin from the pituitary, serum and follicular fluids (Kogawa *et al.* 1991, Krummen *et al.* 1993, Schneyer *et al.* 1992). Double ligand blotting revealed that activin has two binding sites for follistatin, whilst inhibin has only one, thus suggesting follistatin associates with these dimers by way of the common β-subunit (Shimonaka *et al.* 1991). This results in the neutralisation of activin biological activity in some (Asashima *et al.* 1991, Kogawa *et al.* 1991, Xiao *et al.* 1992, de Winter *et al.* 1996) but not all biological systems (Mather *et al.* 1993). Follistatin gene product processing showed that there are two main variants of follistatin resulting from alternative mRNA splicing giving rise to precursors FS344 and FS317. Following post-translational modification, the mature variants take the form of FS315 and the carboxy-truncated variant FS288 (Shimasaki *et al.* 1988, Fukui *et al.* 1993). Full-length FS can undergo further proteolytic cleavage resulting in FS300 (Inouye *et al.* 1991) or FS303 (Sugino *et al.* 1993). Six isoforms of follistatin have so far been purified from porcine follicular fluid, which vary not only in their truncation of the c-terminus, but also in the presence of carbohydrate chains (Sugino *et al.* 1993). FS has been shown to have high affinity for heparin and heparan sulphate chains of cell surface proteoglycans (Nakamura *et al.* 1991). The shortest form, FS288, has the highest affinity for cell surface proteoglycans on rat granulosa cells. By contrast, the longest form FS315 has negligible affinity, and FS303 has only weak affinity for rat granulosa cell surface (Sugino *et al.* 1993). The biological properties of the various isoforms of follistatin were found to be similar in relation to specific activity for activin (Inouye *et al.* 1991, Sugino *et al.* 1993), however FS288 has been shown to have greater biological potency as a rat pituitary cell

culture FSH secretion suppressor than FS315 and FS303 (Michel *et al.* 1993, Inouye *et al.* 1991, Sugino *et al.* 1993). This is probably due to FS288 having a higher affinity for the cell surface and may prevent activin associating with its cell surface receptors (Nakamura *et al.* 1991, Sugino *et al.* 1993). Interestingly, it has been shown that the heparin-binding region of FS is involved in binding activin (Sumitomo *et al.* 1995).

2.2 Implications of Molecular Structure for Immunoassay Development

Several studies have detected the presence of mRNAs for inhibin, activin and follistatin whilst others have used antibodies to localise the expression of the inhibin-α and inhibin/activin β_A and β_B subunits (Baird & Smith 1993, Tuuri *et al.* 1994). However, the presence of mRNA at a specific location does not prove that the actual protein is synthesised there. Furthermore the presence, for example of the β_A-subunit in a cell, could indicate the presence of inhibin-A, activin-A or activin-AB. Only immunoassay can determine into what molecular structure the subunits assemble. The specific measurement of the dimeric molecules of inhibins A and B, and activins A, AB and B was hindered by the lack of specific antibodies to use in the development of sensitive assays, which in turn restricted the development of knowledge of their respective physiological roles. The principle problems which arise during the development of meaningful assays for this protein family include:

- The high degree of homology between the different β subunits.
  This makes it difficult to raise antibodies of adequate specificity.
- The inter-species conservation of the subunits.
  This makes it difficult to raise high affinity antibodies.
- The existence of precursor forms of inhibin and activin.
  This makes it difficult to develop immunoassays specific for defined single molecular forms.

- The existence of free α subunits.
  In certain assay formats, the α subunits can interfere.
- The interference attributable to binding proteins.
  This can lead to poor recovery.

2.3 Previous Assays

2.3.1 Bioassays

In vitro bioassays for inhibin activity based upon the suppression of FSH secretion from both sheep and rat pituitary cells were initially a popular method in the characterisation of inhibin (de Jong *et al.* 1979, Eddie *et al.* 1979, Scott *et al.* 1980, Baker *et al.* 1981, Tsonis *et al.* 1986). Other studies on postnatal rat ovaries saw the induction of FSH-responsive progesterone production after the addition of activin to cultures of ovarian cells (Drummond *et al.* 1996). Further, following the discovery that activin induces erythroid differentiation in the K562 human erythroleukaemia cell line (Yu *et al.* 1987), Schwall & Lai (1991) described a bioassay which was based on activin's ability to stimulate haemaglobin production by this cell line. However one significant problem with this assay is there are at least 19 agents which can induce erythroid differentiation from this line which would make the interpretation of the data difficult. A similar major disadvantage, common to the other assays, is that although they indicate actual bioactivity, previous inhibin/activin bioassays (de Jong *et al.* 1979, Eddie *et al.* 1979, Krummen *et al.* 1993, Sadatsuki *et al.* 1993, Sakai *et al.* 1992) and protein-binding assays (Demura *et al.* 1992, Demura *et al.* 1993) are not specific for inhibin or activin. Cells under investigation for activin expression may co-express biologically opposing inhibin resulting in a "global average" leading to difficulties in the accurate quantification from the data accumulated. The bioassays are also time-consuming and most systems generally lack the sensitivity required for the measurement of serum inhibin obtained under normal physiological conditions (de Jong 1988).

A summary of the characteristics of currently available immunoassays for inhibin α subunits, dimeric inhibins, activins and follistatins are presented in Tables 2.1, 2.2, 2.3 and 2.4 respectively.

Table 2.1 Immunoassays for inhibin α-subunit containing molecules. EIA = enzyme immunoassay; RIA = radioimmunoassay.

Assay Format	Sensitivity	Reference	Antibodies Used	Standard Used
RIA[1]	0.36 ng/ml	McLachlan *et al.* 1986	Rabbit polyclonal	Purified bovine 31 kD inhibin
RIA[2]	~2 ng/ml	Knight *et al.* 1989	Sheep polyclonal	Highly purified bovine 32 kD inhibin
RIA[3]	3.1 ng/tube	Hamada *et al.* 1989	Rabbit polyclonal	Research standard of porcine inhibin
EIA[4]	0.040 ng/ml	Poncelet & Franchimont 1994	Goat polyclonal Mouse monoclonal	Purified human inhibin
EIA[5]	0.078 ng/ml	Baly *et al.* 1993	Chicken polyclonal	rh-inhibin-A
RIA[6]	~1 ng/ml	Lambert-Messerlian *et al.* 1994a	Sheep polyclonal	Peptide used to raise antibody
RIA[7]	~1 ng/ml	Lambert-Messerlian *et al.* 1995	Rabbit polyclonal	Peptide used to raise antibody
RIA[8]	~1 ng/ml	Lambert-Messerlian *et al.* 1995	Sheep polyclonal	Peptide used to raise antibody
EIA[9]	0.005 ng/ml	Groome *et al.* 1995	Mouse monoclonal	Purified human pro-α_C

Radioimmunoassay 1 (RIA[1]) is the original "Monash" assay which has subsequently been found to measure α-subunit containing molecules including free α-subunit. Assays 2 and 3 (RIA[2] and RIA[3]) also measure α-subunit containing compounds. Enzyme immunoassay 4 (EIA[4]) is distributed by Biosource International. This assay and assay 5 (EIA[5]) both cross-react significantly with inhibin-A, inhibin-B and free α-subunit. Assays 6, 7 and 8 (RIA[6], RIA[7] and RIA[8]) all use antibodies raised to synthetic peptides. RIA[6] and RIA[7] measure pro-α inhibin containing precursor forms. Assay 8 (RIA[8]) recognises pro-α inhibin immunoreactive proteins containing the α_N segment of the α-inhibin precursor forms. All of these assays have the potential to detect large dimers of inhibins A and B as well as free α.

2.3.2 Inhibin immunoassays

2.3.2.1 Inhibin α-subunit immunoassays

Despite the development of several immunoassays for inhibin α-subunit containing molecules (Table 2.1), the majority of early studies of human inhibin physiology relied upon one particular radioimmunoassay using a polyclonal antibody raised to purified bovine 31 kD inhibin, and iodinated bovine 31 kD inhibin as tracer. This assay, widely referred to as the "Monash Assay" (McLachlan *et al.* 1986) provided a tool for numerous clinical and physiological studies (Burger 1993). However, it gradually became apparent that it was unable to discriminate between dimeric bioactive inhibin forms and various forms of the free α-subunit which occur in large amounts in body fluids (Robertson *et al.* 1989, Schneyer *et al.* 1990). It became popular in publications to refer to the material measurable by the Monash assay as "immunoreactive" inhibin to acknowledge the possibility that the assay might not accurately measure the levels of bioactive inhibin. It can be seen from Table 2.1 that the two-site ELISA (enzyme-linked immunosorbent assay) for pro-α_C developed by our laboratory is the most sensitive of this group of assays.

2.3.2.2 Dimeric inhibin-A and inhibin-B immunoassays

Several groups prepared monoclonal and polyclonal antibodies with a view to developing two-site immunoassays to measure specifically the dimeric forms of inhibin (Table 2.2). The Genentech group successfully developed several assays using antibodies raised to recombinant inhibins and activins (Baly *et al.* 1993). However, probably because inhibin is closely conserved between species, the antibodies made had low affinity. Thus, these antibodies seemed unable to support ELISAs able to detect the picogram per ml concentrations in which inhibin circulates during the normal menstrual cycle. Franchimont and collaborators also produced two-site assays for various inhibin forms including free α forms, inhibins A and B (Poncelet & Franchimont 1994). These used antibodies raised to synthetic peptides. However, little subsequent application of these assays to clinical material

Table 2.2 Immunoassays for dimeric inhibins. IRMA = immunoradiometric assay; ICL = immunochemiluminescent assay.

Assay Format	Sensitivity	Reference	Antibodies Used	Standard Used
IRMA	0.225 ng/ml	Knight *et al.* 1991	Sheep polyclonal Mouse monoclonal	Highly purified bovine 32 kD inhibin
EIA	0.005 ng/ml	Groome 1991	Mouse monoclonal	rh-inhibin-A
IRMA	0.1 ng/ml	Vaughan & Vale 1993	Sheep polyclonal Rabbit polyclonal	rh-inhibin-A
EIA	1 ng/ml	Baly *et al.* 1993	Mouse monoclonal Chicken polyclonal	rh-inhibin-A
EIA	1 ng/ml	Betteridge & Craven 1991	Mouse monoclonal	Purified bovine 32 kD inhibin
EIA	0.08 ng/ml	Poncelet & Franchimont 1994	Goat polyclonal Mouse monoclonal	Purified human inhibin
ICLA	0.01 ng/ml	McConnell *et al.* 1996	Mouse monoclonal	rh-inhibin-A
EIA	0.002 ng/ml	Groome *et al.* 1994	Mouse monoclonal	rh-inhibin-A
EIA	0.015 ng/ml	Groome *et al.* 1996	Mouse monoclonal	rh-inhibin-B

appears to have been published. By contrast, the assays developed in our laboratory for inhibin-A (Groome *et al.* 1994) and inhibin-B (Groome *et al.* 1996) have been used by many different groups, as detailed in a later section. The main feature of these assays is their high sensitivity and specificity (Table 2.2).

2.3.3 Activin immunoassays

Activin concentrations have been measured in biological material using RIAs (Table 2.3). Shintani *et al.* (1991) developed an activin-A assay which used a polyclonal antibody raised to recombinant material. However, this assay did not take into consideration the interference of activin-binding proteins,

Table 2.3 Immunoassays for activin.

Specific for Total or Free Activin	Assay Format	Sensitivity	Reference	Antibodies	Standard Used
Free	RIA	0.6 ng/ml	Shintani *et al.* 1991	Rabbit polyclonal	Recomb activin-A
Free	RIA	~1.6 ng/ml	Robertson *et al.* 1992	Sheep polyclonal	Recomb activin-A
Free	EIA	0.1 ng/ml	Groome 1991	Mouse monoclonal	Recomb activin-A
Free	EIA	0.2 ng/ml	Wong *et al.* 1993	Mouse monoclonal	Recomb activin-A
Free	EIA	0.4 ng/ml	Wong *et al.* 1993	Mouse monoclonal	Recomb activin-B
Total	RIA	~1 ng/ml	McFarlane *et al.* 1996	Sheep polyclonal	Recomb activin-A
Total activin-B	EIA	no validation paper	Bläuer *et al.* 1996	Mouse monoclonal	Pregnant human serum pool
Total activin-A	EIA	0.05 ng/ml	Knight *et al.* 1996	Mouse monoclonal	Pooled bFF calibrated against recomb activin-A
Total activin-AB	EIA	0.19 ng/ml	Evans *et al.* 1997	Mouse monoclonal	Pooled bFF calibrated against purified porcine activin-AB

nor was it clear whether the assay cross-reacted with other isoforms of activin. Another conventional RIA, developed by Robertson *et al.* (1992), met with limited success when it was used to measure activin in biological fluids. Recently, McFarlane *et al.* (1996) described amendments to the RIA developed by Robertson *et al.* (1992) to measure activin in biological fluids. A dissociating solution was used to disrupt activin-follistatin complexes. However, it was not made clear whether this assay was specific for

activin-A or whether it cross-reacts with the closely related activin B and activin AB dimers. The assay for activin-A developed by our collaborators Knight & Muttukrishna used a novel heat treatment with detergent to irreversibly inactivate activin-binding proteins (Knight *et al.* 1996). A similar assay was subsequently developed for activin-AB (Evans *et al.* 1997). Thus both of these two assays measure "total" activins A or AB in samples (Table 2.3). The ELISA for activin-A has been extensively used, but so far activin-AB has only been found in follicular fluid. Application of an ELISA for activin-B has been described by Bläuer *et al.* (1996), but so far no validation paper has appeared and the reagents are not readily available.

2.3.4 Follistatin immunoassays

Previous assays for follistatin were either in RIA or IRMA format (Table 2.4). Not only were these assays of moderate sensitivity, they also had other disadvantages including the use of hazardous short-lived isotopic reagents (Sugawara *et al.* 1990, Klein *et al.* 1991, Gilfillan & Robertson 1994, Khoury *et al.* 1995, Wakatsuki *et al.* 1996). All of the assays except that of Wang *et al.* (1996) used polyclonal rather than monoclonal antibodies. Wang *et al.* make the claim that their assay is specific only for free follistatin. The evidence for this however is in our view questionable since it is based on a decrease in immunoreactivity observed when an excess of activin was added to recombinant follistatin. It appeared to require a very large molar excess of activin to decrease the signal, whereas it is known from previous studies that only a modest excess of activin is needed to completely complex follistatin (Sugino *et al.* 1993). An alternative explanation of the findings of Wang *et al.* would be that the activin is merely competing with one of the antibodies used in the assay. Thus the follistatin may only become free during the immunoassay.

The main feature of the follistatin assay from our laboratory (Evans *et al.* 1998) is its ability to measure total follistatin with high sensitivity (Table 2.4)

Table 2.4 Immunoassays for follistatin.

Specific for Total or Free Follistatin	Assay Format	Sensitivity	Reference	Antibodies Used	Standard Used
Total	IRMA	0.5 ng/ml	Wakatsuki *et al.* 1996	Mouse monoclonal Rabbit polyclonal	rh-FS 315
Free	IRMA	0.5 ng/ml	Wang *et al.* 1996	Mouse monoclonal	rh-FS 288
Total	RIA	2.5 ng/ml	Gilfillan & Robertson 1994	Rabbit polyclonal	bFS 35 kDa
Total	RIA	4.0 ng/ml	Khoury *et al.* 1995	Mouse polyclonal	rh-FS 288
Total	RIA	1.6 ng/ml	Klein *et al.* 1991	Rabbit polyclonal	bFS 35 kDa
Total	RIA	0.92 ng/ml polyclonal	Sugawara *et al.* 1990	Rabbit	pFS 32, 35, 39 kDa mixture
Free	ICLA	1 ng/ml	McConnell *et al.* 1998	Mouse monoclonal	rh-FS 288
Total	EIA	0.019 ng/ml against	Evans *et al.* 1998	Mouse monoclonal (calibrated	Immunopurified human follistatin rh-follistatin 288)

In the following sections, we describe our strategies for the production of monoclonal antibodies to inhibin/activin subunits, and their use in immunoassay development.

2.4 Preparation of Monoclonal Antibodies to Inhibin

Due to the high homology between the β_A and β_B subunits, the chosen route of antibody production was through the use of synthetic peptides. This method is reliant upon the peptide mimicking one of the native protein's

epitopes resulting in the anti peptide antibody produced having a very high cross-reactivity with the target protein.

Antipeptide monoclonal antibodies hold several advantages over monoclonal antibodies raised to the whole protein molecule.

(1) Raising specific high-affinity monoclonal antibodies to closely conserved molecules is difficult. For example, the β_A and β_B subunits of the different inhibin forms show high homology. Therefore, trying to raise antibodies specific to either inhibin-A or inhibin-B would be very difficult if immunising with the whole protein molecule. Choosing synthetic peptides which correspond to regions where the β_A and β_B subunits show considerable differences in amino acid sequence maximises the chances of generating antibodies specific for each.

(2) Antibodies raised to a whole protein molecule may be specific for conformational epitopes which could be lost during denaturation treatments. Antipeptide antibodies are more likely to recognise their target molecule following denaturation treatments as they recognise continuous epitopes along the primary sequence. As will be seen later, these denaturation treatments proved invaluable in the present work in order to remove interference caused by binding proteins.

(3) If the amino acid sequence of a low-abundance protein of interest or a new protein whose amino sequence has been deduced only from a nucleic acid sequence, synthetic peptides may be the only immediate route to the preparation of useful antibodies.

(4) Antipeptide antibodies also have the advantage of having pre-determined specificity, i.e. it can be assumed that they react with the corresponding protein at a defined point along the primary sequence.

The conformational exposure and associated flexibility of amino-(N) and carboxy-(C) terminal regions of proteins renders analogous synthetic

peptide sequences successful in the production of antipeptide antibodies. Synthetic peptides corresponding to the N- or C-terminus of a native protein are usually of the 15-mer size (15 amino acids residues). Peptides alone act as haptens *in vivo* and therefore do not elicit an immune response from the animal. Instead, it is necessary to couple the peptide to a carrier protein which stimulates the involvement of T-helper cells and therefore enhances the immune response. Carrier proteins commonly employed include keyhole limpet haemocyanin (KLH), bovine serum albumin (BSA), ovalbumin and tuberculin.

2.4.1 Choice of peptide immunogen

Several strategies have been devised for B-cell epitope prediction including:

- hydrophilicity scale (Hopp & Woods 1981) — based on the premise that epitopes are accessible to the solvent and therefore these regions are more likely to be hydrophilic.
- accessibility scale — based on the determination of surface accessibility of each amino acid in various globular proteins (Lee & Richard 1971).
- antigenicity scale (Welling *et al.* 1985) — based on the frequency with which amino acids are present within the epitopes of proteins of deduced antigenic configuration.
- flexibility scale (Karplus & Schulz 1985) — based on temperature factors (B) of selected proteins with recognised three-dimensional structures, being proportional to the root mean square displacement of an atom about an equilibrium position.
- structure — certain characteristics of the secondary structure of proteins such as helices (Hopp 1986) or turns (Krchnak *et al.* 1989, Pellequer *et al.* 1993) have previously been used in the identification of regions of possible antigenicity.

Pellequer & Westhof (1993) devised a program (PREDITOP) which is able to calculate and display a protein profile by using the sequence of the protein together with a propensity scale comprising of hydrophilicity, accessibility, antigenicity, flexibility and turns. This is the programme we used to aid us in deciding which sequences to synthesise and use as the peptide immunogens.

2.4.2 Peptide sequences selected as immunogens

Table 2.5 shows the peptide sequences used to produce the key monoclonal antibodies used in our ELISAs, and Fig. 2.1 shows the epitope locations of each antibody on each subunit.

Table 2.5 Synthetic peptide sequences used to raise monoclonal antibodies.

Subunit	**Peptide Sequence**
α-subunit	NH_2-STPLMSWPWSPSALRLLQRPPEEPAAHANCHR-COOH *Reason chosen*: amino terminal of mature α_C. Used to make monoclonal antibody R1 (Groome *et al.* 1990).
β_A-subunit	NH_2-VPTKLRPMSMLYYDDGQNIIKKDIQNMIVEEC-COOH *Reason chosen*: hydrophilicity and turn predictions. Sequence divergence from β_B-subunit. Used to make monoclonal antibody E4 (Groome & Lawrence 1991).
β_B-subunit	NH_2-IPTKLSTMSMLYFDDEYNIVKRDVPNMIVEEC-COOH *Reason chosen*: hydrophilicity and turn predictions. Sequence divergence from β_A-subunit. Used to make monoclonal antibodies C5 (Groome *et al.* 1996) and 12/13 (Evans *et al.* 1997).
pro region of α-subunit	NH_2-CQGLELARELVLAKVNRALFLDALGPPAVTREGGDPGVRRLPRR-COOH *Reason chosen*: entire region of human inhibin α-subunit. Used to make monoclonal antibody INPRO (Groome *et al.* 1995).

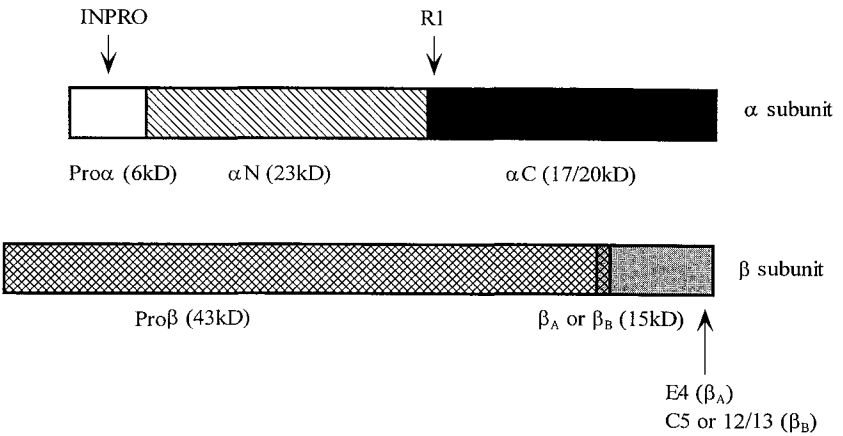

Fig. 2.1 Location of epitopes for monoclonal antibodies used in Oxford Brookes ELISAs.

2.5 The Oxford Brookes ELISAs

In the following section, we describe the immunoassays developed in this laboratory for various inhibin, activin and follistatin forms, and the purposes for which they have been used. These assays all have the following features:

- Use of monoclonal rather than polyclonal antibodies ensuring long-term consistency of assay performance.
- Use of an ultrasensitive detection system with alkaline phosphatase as the label. The substrate-amplification system relies on the alkaline phosphatase to convert the NADPH, present in the substrate solution, to NADH (Self 1985). The NADH then activates a secondary enzyme system which comprises of a redox cycle driven by diaphorase and alcohol dehydrogenase. NADH, in the presence of diaphorase, reduces a tetrazolium salt to form an intensely coloured dye and is itself oxidised to NAD^+. NAD^+ is then reduced to NADH and ethanol is oxidised to acetaldehyde in the presence of alcohol dehydrogenase. The rate of reduction of the tetrazolium salt is directly proportional to the concentration of NADH originally formed by the alkaline phosphatase conjugated to either the detection antibody, or the

streptavidin (depending on each particular assay). Antibody-alkaline phosphatase enzyme conjugates are stable for many years when stored at +4°C.

- All the assays are of two-site (sandwich) format. This has advantages over competitive (RIA-type) assays in terms of specificity and sensitivity (Ekins 1997).

2.5.1 Pro-α_C ELISA

This ELISA uses INPRO monoclonal antibody (Groome *et al.* 1995) as capture antibody and the Fab′ fragment of R1 monoclonal antibody (Groome *et al.* 1990) for detection (Fig. 2.2).

The profile of inhibin isoforms measured by the pro-α_C assay is very similar to those measured by the Monash assay. In human serum and follicular fluid, pro-α_C and pro-α_N-α_C probably account for most of the immunoreactivity observed. Due to the location of the epitopes for R1 and INPRO, the pro-α_C ELISA has the potential to detect all large-form inhibins which possess a pro-region and an α_C-region regardless of whether a

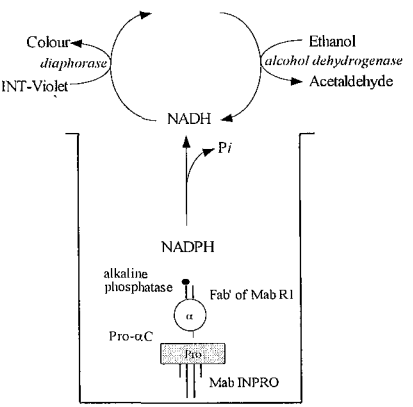

Fig. 2.2 A diagrammatic representation of the pro-α_C ELISA using INPRO as the capture antibody and the Fab′ fragment of R1 coupled to alkaline phosphatase as the detection antibody.

β-subunit or even a pro-β-subunit is present. However, a recent publication by Robertson *et al.* (1997) describes how in practice this pro-α_C ELISA primarily detects monomeric α-subunit forms in serum, with little evidence for the detection of higher molecular weight forms of dimeric inhibin.

The physiological relevance of these forms of the α-subunit remains unclear and in most cases the concentrations of circulating inhibins A or B provide the most useful information. A good example of this is the study by Wallace *et al.* (1997a) of the effects of chemotherapy on testicular function in men. It was found that as sperm count decreased, serum inhibin-B concentrations fell in parallel, and this was accompanied by a rise in serum FSH. The reciprocal relationship between inhibin-B and FSH was consistent with the predictive negative feedback role of inhibin-B. By contrast, immunoreactivity detected by the Monash assay and our pro-α_C ELISA were both elevated in the same samples.

In situations where inhibin bioactivity is important, it is unlikely that the pro-α_C ELISA will be useful for physiological or clinical purposes. However, some epithelial ovarian cancers have been reported to secrete immunoreactive inhibin detectable by the Monash assay (Healy *et al.* 1993). For this application it appears that the less specific the assay the better, preferably measuring *total* α-subunit (Burger *et al.* 1996). Thus it might be anticipated that the pro-α_C ELISA might have some value in monitoring epithelial ovarian cancer. This has been confirmed by Lambert-Messerlian *et al.* (1997) who showed that the combination of pro-α_C and CA 125 levels were more useful as serum markers for epithelial ovarian cancers than CA 125 lone.

2.5.2 Inhibin-A ELISA

The inhibin-A ELISA uses monoclonal E4 (Groome & Lawrence 1991) as the capture antibody and the Fab′ fragment of monoclonal R1 (Groome *et al.* 1990), conjugated to alkaline phosphatase, as the detection antibody (Fig. 2.3).

There have been three independent validation studies for the inhibin-A (Groome *et al.* 1994, Muttukrishna *et al.* 1994, Lambert-Messerlian *et al.* 1994b). In 1994, Knight & Muttukrishna made an interesting discovery that

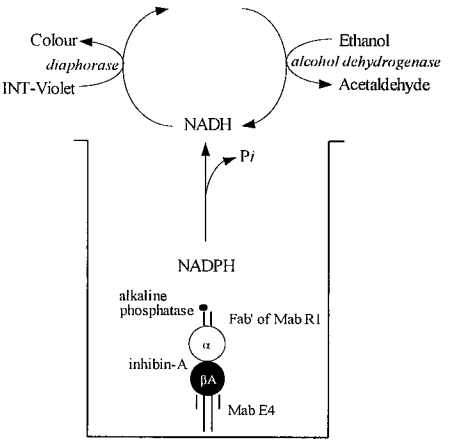

Fig. 2.3 A diagrammatic representation of the inhibin-A ELISA using E4 as the capture antibody and the Fab′ fragment of R1 coupled to alkaline phosphatase as the detection antibody.

the immunoreactivity of inhibin with our E4 monoclonal antibody was increased on long-term storage of the sample. This was shown to be due to the spontaneous oxidation of methionine to the sulphoxide during long-term storage. The epitope for the E4 antibody contains the sequence MSM (see Table 2.5). A preassay oxidation step was therefore introduced into the inhibin-A assay resulting in improved sensitivity (Knight & Muttukrishna 1994, Groome *et al.* 1994). This step merely requires addition of hydrogen peroxide (Knight & Muttukrishna 1994). It is important to avoid the use of haemolyzed samples as the catalase present will reduce the effectivness of the hydrogen peroxide and lead to an underestimate of the concentration of inhibin in that sample.

The currently recommended version of the assay now has an additional preassay treatment involving the addition of detergent (SDS), and a heat treatment (Wallace *et al.* 1998a). This step was first introduced into the activin-A assay developed by Knight *et al.* (1996) using our E4 antibody. In this assay it was essential to denature follistatin and allow detection of

total activin. The advantages of using the SDS heat treatment for inhibin-A assay are:

- The treatment destroys catalase enabling valid inhibin-A results to be obtained with partially haemolyzed samples.
- The treatment destroys any proteases which may be present, allowing the entire assay to be carried out at room temperature.
- All inhibin and activin assays are susceptible to interference by binding proteins such as follistatin or α2-macroglobulin (α2M) which may mask epitopes on the analyte. This treatment removes any interference due to these proteins, ensuring that the concentration measured corresponds to the "total" amount present.
- All potential false positive-causing agents seem to be removed, and serum samples from postmenopausal, ovariectomized or castrate individuals give uniformly negative results for inhibins A and B as expected.
- After the heat treatment, the high molecular weight forms of inhibin have similar immunoreactivity to the mature forms so that the total inhibin assay values recorded will more closely reflect total bioactive inhibin-A than if the heat treatment is not used. Wallace *et al.* (1998a) produced some data showing that the modified format performs very well when inhibin-A is used as a prenatal screening marker for Down's syndrome.

As well as being used to measure the serum concentrations of inhibin-A in women during the normal menstrual cycle (Groome *et al.* 1994, Yamoto *et al.* 1997), the assay has been used for many other studies. Possible clinical applications of the inhibin-A assay include its use in prenatal screening for Down's syndrome (Wallace *et al.* 1995, Cuckle *et al.* 1995, Wallace *et al.* 1996, Aitken *et al.* 1996, Watt *et al.* 1996, Wenstrom *et al.* 1997, Wald *et al.* 1998), monitoring of ovarian granulosa cell tumours (Cooke *et al.* 1995, Burger and Fuller 1996), prenatal screening for pre-eclampsia (Muttukrishna *et al.* 1997, Cuckle *et al.* 1998) and for monitoring women undergoing IVF (Lockwood *et al.* 1996). Blumenfeld *et al.* (1998) have also

shown that serum inhibin-A concentrations may serve as a prognostic indicator of the resumption of ovarian function in young women following chemotherapy. As well as the large number of studies on serum or plasma, several groups have used the inhibin-A ELISA to study levels in other fluids such as amniotic fluid (Wallace *et al.* 1997b, Wallace *et al.* 1997c, Riley *et al.* 1996, Muttukrishna *et al.* 1999) and ovarian follicular fluid (Magoffin & Jakimiuk 1997, Lau *et al.* 1999).

2.5.3 Inhibin-B ELISA

The inhibin-B ELISA uses C5 monoclonal antibody (Groome *et al.* 1996) as capture antibody and the Fab′ fragment of R1 monoclonal antibody (Groome *et al.* 1990) for detection (Fig. 2.4).

In common with the inhibin-A ELISA, sample pre-treatment for inhibin-B assay is carried out with SDS/heat and hydrogen peroxide (Groome *et al.* 1996). The SDS heat treatment was found to be particularly important in the inhibin-B assay to remove a false positive signal found in many

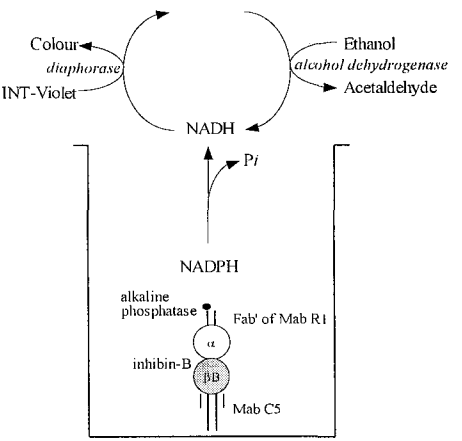

Fig. 2.4 A diagrammatic representation of the inhibin-B ELISA using C5 as the capture antibody and the Fab′ fragment of R1 coupled to alkaline phosphatase as the detection antibody.

human serum samples. This signal did not seem to be due to conventional heterophile antibodies (Hunter & Budd 1980), and no other method of negating its effects could be found. After the SDS heat treatment, the artifact was totally removed and serum from postmenopausal, ovariectomized or castrate individuals had no measureable inhibin-B.

Whereas circulating inhibin-A is only found in females, inhibin-B is present in the circulation in both men and women. In the menstrual cycle, inhibin-B is produced by small follicles at the start of the cycle, and may be involved in regulating the inter-cycle rise in FSH (Groome *et al.* 1996). It has been shown that as women age, their serum FSH levels in early follicular phase rise, and their serum inhibin-B levels fall (Klein *et al.* 1996). Serum inhibin-B has been proposed as a marker for ovarian reserve, and for predicting the likelihood of a successful outcome to IVF (Seifer *et al.* 1997, Sharara *et al.* 1998). Patients with polycystic ovarian disease have been found to have elevated serum inhibin-B concentrations and to lack the pulsatile pattern found in normal individuals (Lockwood *et al.* 1998). Petraglia *et al.* (1998) found that inhibin-B is the major form of inhibin secreted by granulosa cell tumours.

Studies of inhibin secretion in the human male using previous assays have yielded confusing results. No negative relationship was found between immunoreactive inhibin (measured by the Monash and similar assays) and FSH. This led some groups to conclude that inhibin was unimportant in the human male (Lambert-Messerlian *et al.* 1995). By contrast, when it became possible to measure serum inhibin-B levels, a strong negative relationship between inhibin-B and FSH was found (Illingworth *et al.* 1996). There is now general agreement that inhibin-B is a useful circulating marker for spermatogensis in individual patients (Klingmuller & Haidl 1997, Anawalt *et al.* 1996, Pierik *et al.* 1998). Inhibin-B also shows potential in population studies of male fertility (Jensen *et al.* 1997) and for the monitoring of testicular damage during chemotherapy (Wallace *et al.* 1997a). Nachtigall *et al.* (1996) used before-treatment serum inhibin-B concentrations to identify individuals with hypogonadism more likely to benefit from GnRH replacement therapy. Both inhibins A and B have potential in the study of hormonal profiles in children with abnormal pubertal development (Crofton *et al.*

1997). Inhibin-B showed little correlation with FSH before puberty but a strong negative relationship thereafter.

2.5.4 Activin-A ELISA

The activin-A immunoassay uses E4 monoclonal antibody (Groome & Lawrence 1991) as both capture and detection antibody. E4 antibody is isotype IgG2b and so cannot be made into an Fab′ alkaline phosphatase conjugate. Therefore, the E4 used for detection is biotinylated and strepatavidin-alkaline phosphatase is incorporated into the assay procedure (Fig. 2.5).

As noted earlier, this ELISA uses two modifications introduced by Knight *et al.* (1996). Firstly, the SDS/heat treatment which denatures follistatin to allow measurement of total activin. Secondly, the hydrogen peroxide pretreatment to improve the immunoreactivity of the activin with the E4 monoclonal antibody used for capture and detection.

Several studies have employed this ELISA to measure activin-A concentrations in human serum. Muttukrishna *et al.* (1996) used the assay

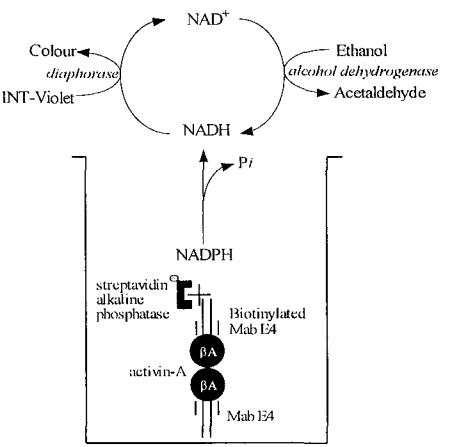

Fig. 2.5 A diagrammatic representation of the activin-A ELISA using E4 as the capture antibody and biotinylated E4 as the detection antibody.

to measure circulating activin-A both during the human menstrual cycle and in pregnancy. Possible clinical applications of this assay include its use in prenatal screening for pre-eclampsia (Muttukrishna *et al.* 1997) and a biochemical marker for oocyte quality (Lau *et al.* 1999). In common with inhibin-A, maternal serum activin-A concentrations are significantly elevated in Down's syndrome pregnancies (Cuckle *et al.* 1999), however the overlap between affected and unaffected pregnancies was too great for it to be practical as a marker for screening.

2.5.5 Activin-AB ELISA

The activin-AB immunoassay uses E4 monoclonal antibody (Groome & Lawrence 1991) as capture antibody and biotinylated monoclonal antibody 12/13 (Evans *et al.* 1997) detection antibody. As in the activin-A assay, streptavidin-alkaline phosphatase is incorporated into the procedure (Fig. 2.6), and a heat/SDS and hydrogen peroxide pretreatment step is included in the procedure.

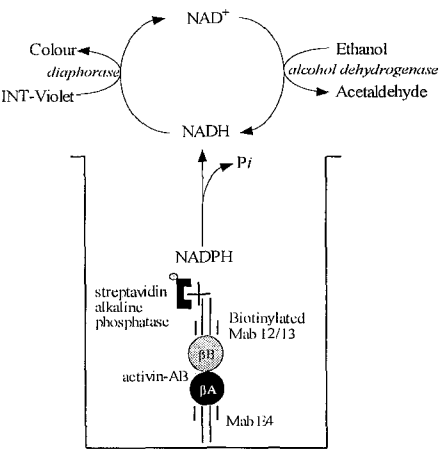

Fig. 2.6 A diagrammatic representation of the activin-AB ELISA using E4 as the capture antibody and biotinylated 12/13 as the detection antibody.

The distribution of activin-AB appears to be extremely restricted compared to that of activin-A. The relatively high concentrations of activin-AB in ovarian follicular fluid supports a role in follicular development (Evans *et al.* 1997). In all other samples tested, such as maternal serum samples throughout pregnancy (Fowler *et al.* 1998), seminal plasma samples (Anderson *et al.* 1998), placental extracts and serum samples from postmenopausal, hyperstimulated and normal cycling women (Evans *et al.* 1997), there were undetectable levels of activin-AB.

2.5.6 Follistatin ELISA

The follistatin immunoassay uses monoclonal antibody 29/9 (Evans *et al.* 1998) as capture antibody and the Fab′ fragment of monoclonal antibody 17/2 (Evans *et al.* 1998) as detection (Fig. 2.7).

The diluent used in this assay includes sodium deoxycholate and Tween 20 which disrupt follistatin-activin complexes in samples, rendering the assay able to measure total follistatin. Similar to the assay developed by Wang

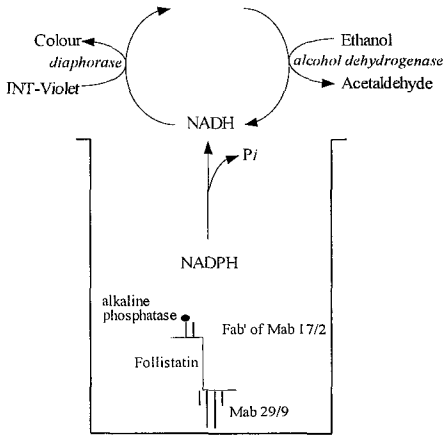

Fig. 2.7 A diagrammatic representation of the follistatin ELISA using 29/9 as the capture antibody and the Fab′ fragment of 17/2 coupled to alkaline phosphatase as the detection antibody.

et al. (1996), this assay only recognises human follistatin, suggesting that the epitope of either one or both monoclonal antibodies includes the uniquely human 171–175 amino acid region.

No consistent trend in serum follistatin levels throughout the normal human menstrual cycle was found (Evans *et al.* 1998). However, the levels during pregnancy increased in parallel with activin-A (Fowler *et al.* 1998). Amniotic fluid follistatin levels decrease as pregnancy progresses but do not differ between normal and Down's syndrome pregnancies (Evans *et al.* 1998, Muttukrishna *et al.* 1999, Wallace *et al.* 1999). The assay has also been used to measure follistatin production by the male reproductive tract (Anderson *et al.* 1998), and concentrations present in extra-embryonic coelomic and amniotic fluids in early pregancy (Riley *et al.* 1998).

Compared to follistatin-288, follistatin-315 significantly cross-reacts in this assay (9.9%). A recent study suggested that follistatin-315 is the dominant form of follistatin in serum, with follistatin-288 being the major form in follicular fluid (Schneyer *et al.* 1996). If this is the case, then we would expect our assay to measure 10% of the true value present in serum samples. This would explain the differences in female serum concentrations of ~8 ng/ml found by Khoury *et al.* (1995) and 12.5 ng/ml found by Wakatsuki *et al.* (1996) compared to our findings of 0.5–1.0 ng/ml. At present it is not known which forms of follistatin exist in different tissues and fluids, thus it is possible that mixtures may be found. However, the high starting sensitivity of this assay compensates for the reduced sensitivity for serum forms.

2.6 Assay Validation

Once an assay has been developed and optimised, it then needs to be validated in order to confirm its exact characteristics. Only when a validation paper has been published can users be confident in using the assay for their studies. The validation of our inhibin-A (Groome *et al.* 1994), inhibin-B (Groome *et al.* 1996), pro-α_C (Groome *et al.* 1995), activin-A (Knight *et al.* 1996), activin-AB (Evans *et al.* 1997) and follistatin (Evans *et al.* 1998) ELISAs described here have been published. A recent publication (Wadhwa & Thorpe

1998) outlined the factors to be investigated during assay validation. In the following section, we will comment on each of these factors in relation to our assays.

2.6.1 Specificity

In an ideal situation, the analyte would be a single molecular species, identical to the available purified standard. The assay would show no significant cross-reactivity with other molecules found in samples. This situation is rarely achievable for protein hormones, and is almost unimaginable for the closely-related inhibin family of molecules. For example, inhibin-A in body fluids consists of a mixture of isoforms in which α and β_A subunits of various lengths, combine to form various size isoforms of bioactive inhibin-A. Two-site inhibin-A assays, such as our ELISA described below, recognise most, if not all, of these isoforms to varying extents (Robertson *et al.* 1996, McConnell *et al.* 1996). When this assay was designed, it had to achieve two main specificity goals. One important specificity requirement for this assay was an inability to recognise forms of the α-subunit unassociated with a β-subunit, e.g. pro-α_C. Thus, by contrast to the previously used "Monash" radioimmunoassay, the inhibin-A ELISA only measures $\alpha\beta$ dimers, the bioactive forms. The second specificity requirement, was an ability to distinguish inhibin-A from inhibin-B. This ability is totally dependent on the high specificity of the E4 monoclonal antibody for the β_A-subunit.

Other sources of protein analyte heterogeneity which can complicate immunoassay are glycosylation (Storring 1992) and partial proteolysis. At the present time, no data is available on whether glycosylation variants of inhibin are equally immunoreactive, or if some of the bioactive inhibin-A in serum samples is non-immunoreactive due to proteolysis. Although none of the peptide epitopes themselves contain a known glycosylation site, there is still the potential for conformational effects.

Three main forms of follistatin (follistatin-288, follistatin-303 and follistatin-315) are known to exist (Sugino *et al.* 1993). To date, there is little information regarding the relative concentrations of these in various

tissues and biological fluids. If certain tissues or fluids contain only one isoform, then the fact that an assay used to measure it has the potential to cross-react with other forms is of little concern. Problems arise when the assay is used to measure follistatin concentrations in samples which contain a mixture of forms. Here, precise quantification is impossible. With the exception of our publication detailing the development of our follistatin assay (Evans *et al.* 1998), nobody has tested the reactivity of other follistatin forms in each of the previously developed assays.

Of the assays described here only two have any practical limitations in respect of specificity, i.e. inhibin-B and follistatin. The monoclonal antibody C5 to the β_B-subunit has a 0.5% cross-reaction with the β_A-subunit. Thus inhibin-A cross-reacts to approximately this extent in the inhibin-B assay (Groome *et al.* 1996). The only situation in which this is likely to cause erroneous conclusions is where a sample has high concentrations of inhibin-A and low concentrations of inhibin-B. In the recent study by Petraglia *et al.* (1998) of inhibin-B in late pregnancy, the apparent low concentrations of inhibin-B found in maternal serum could easily be accounted for by cross-reaction of the very high levels of inhibin-A in these same samples. Our follistatin assay (Evans *et al.* 1998) probably measures all forms of follistatin in the samples tested, even though single preparations of recombinant follistatins 288 and 315 were actually available to test.

2.6.2 Reagent stability and analytical validity

Inter- and intra-assay variation in the ELISAs are dependent on reproducible coating of microplates with antibody, standardised drying procedures and their storage in dessicated foil packs. In all the ELISAs, the inter- and intra-plate coefficients of variation should be less than 10%. Manual ELISAs, however are operator-dependent and higher values may be observed with those new to the technique. The coefficient of variation attributable to the plate itself should be less than 5% when coating, drying and storage conditions are optimal. Best results have been obtained by our collaborators when we supply them with foil-packed coated plates.

2.6.3 Interference

Interference can occur due to the presence of several entities including binding proteins, heterophilic antibodies and rheumatoid factors etc. Heterophilic antibodies are a well recognised form of interference in enzyme immunoassays (Hunter & Budd 1980). They are generally directed against non-human IgG antibodies and may contribute to serum-mediated non-specific binding (NSB) by attaching the labelled antibody to the capture antibody. Two studies carried out in the US have displayed the prominence of heterophile antibodies in 30–40% of patient samples (Boscato & Stuart 1986, Boscato & Stuart 1988). Heterophilic antibody activity may be partly eradicated by adding serum or immunoglobulins from the same species as the antibody reagents to the assay matrix (Jones *et al.* 1992). Rheumatoid factors are autoantibodies which are composed of aggregated immunoglobulin molecules, typically IgMs. Similar to heterophilic antibodies, rheumatoid factors are believed to contribute to serum-mediated non-specific binding as a result of cross-linking the Fc fragments of both the capture and the labelled antibody. These undesired effects may be overcomed by using fragmented immunoglobulins, i.e. Fab′ or (Fab′)2 for either or both of the antibodies used, resulting in the elimination of the potential interference site (Weber *et al.* 1990).

In the present inhibin and activin ELISAs, the sample pretreatment involving the addition of SDS and heat eliminates all potential interference from heterophile antibodies, follistatin, α_2-macroglobulin, etc. In the follistatin ELISA, the use of mouse serum and the Fab′ fragment of the detection antibody minimises the effects of heterophile antibodies, and the deoxycholate/Tween 20 assay diluent removes interference from activin.

2.6.4 Matrix effects

An ideal assay matrix would be the same fluid as the sample, but completely devoid of analyte. In our initial studies for the assay of inhibins A and B, we used as matrix the serum from postmenopausal women. Subsequently we found that foetal calf serum gave similar results and was more easily obtained. For the activin, pro-α_C and follistatin assays, the matrix used for the assay

is the diluent. For the activin and follistatin assays, a one in two dilution of the sample gives good recovery of spiked standard. For the pro-α_C assay at least a one in four dilution is required.

2.6.5 Sample collection/storage

Recommendations on sample collection, processing and storage should be provided for any new assay.

Inhibins A and B, activin and follistatin immunoreactivity appears to be stable for long periods of time at –20°C although we recommend storing samples at –70°C to –80°C. Cuckle *et al.* (1994) and Lambert-Messellian *et al.* (unpublished) have produced evidence that the pro-α_C immunoreactivity in serum stored at –20°C may decline by up to 20% per year. The stability of the dimeric inhibins suggests that it is probably the epitope recognised by the INPRO monoclonal antibody which is sensitive to proteolysis on long-term frozen storage. Most of the assays use serum as the sample although results obtained with plasma are closely similar. It is recommended that all sera be removed from the clot the day following blood collection, and that all studies closely comparing analyte concentrations use samples which have been stored in similar fashion for similar times.

2.6.6 Linearity and parallelism

Serial dilutions of samples are analysed to ensure that the dose response curves of samples are parallel to the standard curve. The inhibin activin and follistatin assays all gave dose response relationships identical with those of the standards.

2.6.7 Recovery

This assesses the assay's ability to recover a known amount of analyte which has been "spiked" into a sample. This is to confirm the stability of the analyte in the assay matrix i.e. to ensure that no analyte degradation

occurs during the assay procedure. This analysis also serves to confirm that there is no interference from other sample components such as binding proteins. Another potential cause of poor recovery is if the capacity of the capture antibody is depleted by the presence in certain samples of cross-reactive forms of the analyte recognised by the capture antibody but not the detection antibody. For example, recovery of small amounts of inhibin-A in a solution containing large concentrations of activin-A may be impaired because of the common β_A-subunit. It is possible to configure two-site assays to minimise this problem by using the more specific antibody for capture. In the case of inhibin-A assay, the reason the E4 monoclonal antibody was chosen for capture is that it ignores the large amount of free α often present in many samples. This was judged a much more significant problem than activin interference since there are few samples which will contain large amounts of activin-A and a small amount of inhibin-A.

2.6.8 Sensitivity

The procedure used to calculate the sensitivity of the assay should be clearly documented and is usually defined as the limits of quantification. Porstmann & Kiessig (1992) defined the detection limit of an assay as the lowest dose of analyte which generates an absorbance value equal to x true blank absorbance + 5 × SD of the zero-dose sample.

2.7 Standardisation

The objective of standardisation is to facilitate inter-laboratory comparison. A recent review article on cytokine immunoassays emphasises the important role of assay standards in maintaining consistency from laboratory to laboratory and from kit to kit. The use of a standard identical to the analyte is the ideal, otherwise standardisation in its most strict sense is not possible (Ekins 1991). However, most assays use a standard which contains "the analyte in a form identical to that found in the sample, or similar enough to behave identically in the assay system". The presence of a multiplicity of

molecular forms of inhibin and activin makes it difficult to set up a standard which is applicable to each form of molecule. It is known that inhibin in follicular fluid is present in a variety of molecular sizes (Robertson *et al.* 1997), and that inhibin in serum is also present in a mixture in which the components are not in the same proportions as in follicular fluid. Male and female sera also contain different isoforms. These proportions may also differ under different clinical conditions. As the 32 Kd form of inhibin available in recombinant form is only one distinct molecular structure amongst many in real samples, it cannot be taken for granted that recombinant inhibin is a more appropriate standard than an immunopurified preparation which contains a realistic/native mixture of molecular forms. Possibly the most appropriate standard for a serum assay of inhibins A or B is a real serum sample of similar nature to the patient sample to be tested.

Most researchers are using Genentech recombinant inhibin-A, inhibin-B, activin-A, activin-B and NHPP and NIDDK follistatin-288 (National Hormone and Pituitary Program and National Institute of Diabetes and Digestive, and Kidney diseases) as primary standards. So far, only one recombinant preparation has achieved the status of a WHO (World Health Organisation) standard (Rose & Das 1996). There is a need for similar WHO standards for the other materials. For activins A and B, the recombinant forms are probably already available in sufficient purity and quantity. For activin-AB assay, an interim standard is being used consisting of a one in 50 dilution of pooled bovine follicular fluid. This was calibrated in our laboratory against purified natural porcine activin-AB (a kind gift of Dr. H. Sugino). Limited amounts of Genentech recombinant inhibin-B are currently available and results obtained in several laboratories raises the possibility that some of the preparations may have undergone partial proteolysis at the N-terminal of the α-subunit (Robertson *et al.* 1995). Thus there is little uncertainty about the precise concentration of the recombinant preparations in use. Therefore, to provide an interim standard, we have used an immunopurified concentrate of inhibin forms from human follicular fluid as a standard for our inhibin-B assay and have distributed it to our collaborators. For our pro-α_C assay, we distributed standards based on the human pro-α_C which we purified. At the time of writing this chapter we are presently discussing with the National Institute of Biological Standards (NIBS), the possibility of producing

inhibin-B and pro-α_C immunoassay standards based on our preparations. The commercially available forms of these assays (Serotec Ltd.) use purified pro-α_C as a standard in the pro-α_C assay, immunopurified hFF concentrate for the inhibin-A and inhibin-B assays, and diluted bovine follicular fluid for the activin-A and activin-AB assay. The standard preparation used in our follistatin assay was immunopurified from human follicular fluid. This was calibrated in our laboratory against recombinant human follistatin-288 (a gift from the NHPP and NIDDK).

2.8 Further Assays Needed in This Field

2.8.1 Activin

We have so far not been able to develop an assay for activin-B. Attempts to sandwich C5 with itself (similar to E4 in the activin-A assay) or with 12/13 were surprisingly met with limited success. As yet there is no definite explanation for this. Although sequences for a human β_C- and β_E-subunit have been identified and shown to be expressed in liver, it is not certain at the present time whether these can assemble with the inhibin-α subunit to form inhibins C and E. It has been shown that dimeric activins C and E can be formed in recombinant expression systems, and that these subunits can form a variety of heterodimers with the other activin subunits. Whether any of these forms have a biological function is uncertain. However, immunoassays for different activin isoforms may be useful to evaluate the function of these subunits in the liver. Assays for free (none follistatin-bound) activin would be useful in addition to assays for total such as ours. However, rigorous validation would be needed to show that the immunoassay itself was not dissociating the complexes.

2.8.2 Inhibin

It is known that dimeric inhibins A and B occur in human plasma as a variety of size isoforms (Robertson *et al.* 1995). Although all may be

bioactive, it is possible that the relative proportions of these isoforms vary in disease states. Little is known about the relative proportions in which the different isoforms occur and how these might change in individual patients. In order to address the above issues, we hope to develop specific immunoassays for these size isoforms, using new monoclonal antibodies raised to α_N, pro-β_A and pro-β_B.

2.8.3 Follistatin

Immunoassays specific for follistatin- 303 or 315 are possible. An immunoassay able to measure follistatin-315 specifically in the circulation might be useful, as this isoform is claimed to be the predominant form in blood. However, our follistatin-288 assay measures serum follistatin satisfactorily and no clinical application of follistatin assay has yet been suggested.

2.9 Conclusion

Workers in the inhibin, activin and follistatin field now have access to most of the immunoassay tools needed to explore the functions of this family of molecules. Attention should now be directed towards finding clinical applications for these measurements.

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CHAPTER 3

ENDOCRINE, AUTOCRINE AND PARACRINE ACTIONS OF INHIBIN, ACTIVIN AND FOLLISTATIN ON FOLLICLE-STIMULATING HORMONE

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3.1 Introduction

Follicle-stimulating hormone (FSH) is a key reproductive hormone involved in the regulation of follicular development. Many anovulatory disorders are accompanied by dissociations in luteinising hormone (LH) and FSH release. For instance, patients with polycystic ovarian syndrome (PCOS) show an abnormally elevated ratio of LH/FSH (Ehrmann *et al.* 1995). Similarly, during perimenopause (the transitional phase that leads to complete cessation of cyclicity), increases in FSH secretion often precede LH abnormalities (Sherman & Korenman 1975). An understanding, therefore, of the control of FSH secretion and the mechanisms involved in the differential regulation of FSH from LH release will help elucidate the etiology of these conditions and may provide long-term benefits for enhancement or control of fertility.

3.2 Control of FSH

In spite of its importance and in striking contrast to the wealth of information available regarding LH secretion, our understanding of the regulation of FSH is incomplete. Available evidence suggests that, in contrast to the nearly absolute dependence of the LH secretory system on gonadotrophin-releasing hormone (GnRH) pulsatility (Marshall *et al.* 1992), FSH secretion may be regulated at multiple levels (Chappel 1985, 1989, Chappel *et al.* 1983, McNeilly 1988, McNeilly *et al.* 1995). At the hypothalamic level, GnRH regulates FSH secretion (Marshall *et al.* 1992), although evidence exists to support the existence of a separate FSH-releasing factor (McCann *et al.* 1983, Yu *et al.* 1997). Recent studies utilising the hypophyseal portal approach to monitor FSH secretory profiles have shown that the basal mode of FSH secretion is dominant (Padmanabhan *et al.* 1997a). Seventy per cent of FSH appears to be secreted in the basal mode in the ovariectomised sheep with very little being secreted in the pulsatile component. This is also supported by the finding that a substantial portion of secreted FSH bypasses packaging into secretory granules but rather is secreted directly (Muyan *et al.* 1994, Farnworth 1995). FSH secretion is also tightly coupled to biosynthesis and dependent on the availability of translatable FSHβ mRNA (Farnworth 1995, McNeilly *et al.* 1995, Muyan *et al.* 1994).

In addition to the established FSH-regulatory role of GnRH, several lines of evidence support the existence of a hypothalamic-independent component of FSH secretion: (1) FSH secretion continues for prolonged periods (up to several weeks) in sheep in which hypothalamic inputs to the pituitary are severed (Clarke *et al.* 1983, Hamernik & Nett 1988), (2) release of FSH continues in hypophysectomised rats (DePaolo 1991a) and sheep (Padmanabhan *et al.* 1997b) with pituitary transplants under the kidney capsule, (3) FSH continues to be secreted in long-term pituitary cultures in the absence of GnRH stimulation (Sheridan *et al.* 1979).

3.3 Role of Inhibin, Activin and Follistatin in the Control of FSH

What are the regulatory mechanisms involved in the control of this dominant mode of FSH biosynthesis and release? In addition to the well established,

feedback effects of oestradiol (Phillips *et al.* 1988, Gharib *et al.* 1990, Baird *et al.* 1991), several studies conducted during the last decade have documented the negative influences of inhibin (Baird *et al.* 1991, Carroll *et al.* 1991, Mayo 1995, Weiss *et al.* 1993) and follistatin (Meriggiola *et al.* 1994, Ying *et al.* 1987) and the positive role of activin (Carroll *et al.* 1991, Mayo 1995, Weiss *et al.* 1992, 1993) in regulating the biosynthesis and release of FSH.

Before we assign a physiologic role for these regulators in the control of FSH secretion, it is essential we gain an understanding of the structural and functional overlap that exists among them as well as the sites from which they originate. These regulatory proteins show extensive structural and functional relationships. Activins (A, B and AB) and inhibins (A and B) are members of the TGFβ family of growth and differentiation factors. They were first isolated from gonadal fluids and believed to act predominantly in an endocrine manner to modulate FSH secretion (Ying 1988). The inhibin α, β_A and β_B subunits are encoded by distinct genes and dimerise to give rise to inhibin-A (β_A), inhibin-B (β_B), activin-A ($\beta_A\beta_A$), activin-AB ($\beta_A\beta_B$) and activin-B ($\beta_B\beta_B$) (Ying 1988). In terms of FSH secretion, inhibin and activin are functional opposites, inhibin suppress FSH and activin stimulate FSH (Mather *et al.* 1992, DePaolo *et al.* 1991b, Ying 1988). Follistatin (several variants have been identified; Shimasaki 1988), monomeric protein distinct from both inhibin and activin, exhibit a functional overlap with inhibin in the suppression of FSH release (Ying 1987, 1988, Ying *et al.* 1987). They act as binding proteins for both activins and inhibins (Robertson 1992, Krummen *et al.* 1993, Michel *et al.* 1993). Binding of follistatin to activin completely negates activin action (Cataldo *et al.* 1994, Nakamura *et al.* 1990, Mather *et al.* 1993, de Winter *et al.* 1996). On the contrary, binding of follistatin to inhibin (Shimonaka *et al.* 1991) does not negate inhibin action (Fig. 3.1).

Evidence to date suggests that all of the inhibitory action of follistatin on FSH may be mediated via negation of activin action. Whether follistatin has other direct, independent effects in the control of FSH secretion remains to be determined. α-2 macroglobulin, yet another protein that binds activin and inhibin (Mather *et al.* 1992, Vaughan & Vale 1993, Schneyer *et al.* 1992), does not negate activin or inhibin action. It appears that inhibin,

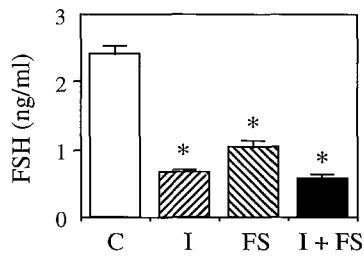

Fig. 3.1 Effects of maximally effective doses of inhibin (I), follistatin (F) and inhibin plus follistatin (I + F) on FSH release from cultured ovine pituitary cells. Ovine pituitary cell monolayers were treated for 72 hours (days 3–5) with I (10 ng), F (10 ng) or I + F. On day 5 of culture, medium was replenished and FSH released during the subsequent 3 hours period (in the continued presence of above treatments) are shown (Cemeroglu *et al.* 1997).

whether in a free or bound state retains its biologic activity whereas activin is only active when it is not bound to follistatin. Adding to this complexity, activin receptors (ActR) exist in multiple forms (ActRI, ActRIB, ActRII and ActRIIB) (Mathews & Vale, 1991, Attisano *et al.* 1992, Mathews, 1994) and the activin signal cascade requires the presence of both type I and II activin receptors (Attisano *et al.* 1992, Mathews, 1994).

There is also evidence that inhibin can antagonise activin stimulation of FSH (Weiss *et al.* 1993, Padmanabhan *et al.* 1995b). Whether such antagonism is mediated via blockade of inhibin binding to activin receptor or inhibin binding to inhibin-specific receptors remains to be determined. *In vitro* studies have shown that inhibin has the ability to bind activin receptor, albeit with lower affinity, and block activin-binding (de Winter *et al.* 1996, Xu *et al.* 1995, Martens *et al.* 1997, Lebrun *et al.* 1997, Antisano *et al.* 1992, Mathews & Vale, 1991). The inability of inhibins to recruit ALK4 following activin receptor binding provides a competitive model for activin antagonism by inhibin. Failure of inhibins to antagonise activin in some tissues and cells suggest that additional components are likely to be involved in inhibin action. While ample evidence exists supporting the existence of inhibin-binding sites (Draper *et al.* 1998, Hertan *et al.* 1999, Woodruff, 1999), it remains to be determined if these binding sites represent "*bona-fide*" inhibin-specific receptors or ancillary-binding proteins that bind the ligand but fail to generate

an intracellular signal. Such binding proteins have been identified for other members of the TGFβ superfamily (Lopez-Casillas *et al.* 1993, Jacques *et al.* 1994, Worby *et al.* 1998, Yamashita *et al.* 1995, Treanor *et al.* 1996). Recent studies have shown that type III TGF-β receptor, betaglycan, functions as an inhibin co-receptor with ActRII (Lewis *et al.* 2000) A specific membrance-anchored proteoglycan (pl20) with affinity for inhibin-A has also been identified in bovine pituitaries (Chong *et al.* 2000). The full-length cDNA of this protein is predicted to be a 1336-amino acid glycoprotein. Assuming that an inhibin receptor exists, it remains to be determined, whether the signal transduction pathway of the putative inhibin receptor converges downstream with the signal transduction pathway of activin receptor. Irrespective of the mode of action, the structural and functional overlap that exists among these regulators dictates that determination of the relative proportion of activin, inhibin and follistatin is an important consideration in determining their overall impact on FSH.

Can one assess the input (net stimulatory or inhibitory input at any given point in time) from these regulatory proteins by merely measuring the individual components in the peripheral circulation? Contrary to the early beliefs that activin, inhibin and follistatin originate at the gonadal level and act in an endocrine manner, more recent studies have shown that these regulatory proteins are also expressed at the pituitary level and may act in an autocrine/paracrine manner to regulate FSH secretion (Mather *et al.* 1992, Bilezikjian *et al.* 1993a, DePaolo *et al.* 1991b, 1993). Therefore, in assessing the net input of these regulatory components, it is important to address their relative contribution, not only at the peripheral (ovarian and other), but also at the pituitary level.

3.3.1 Peripheral Contribution

Recent implementation of dimer-specific assays (Groome *et al.* 1994, McConnell *et al.* 1996, 1998, Poncelet & Franchimont, 1994, Groome *et al.* 1996, Woodruff *et al.* 1993, Knight *et al.* 1996, Khoury *et al.* 1995, Wang *et al.* 1996, Wakatsuki *et al.* 1996) have provided us with the necessary tools

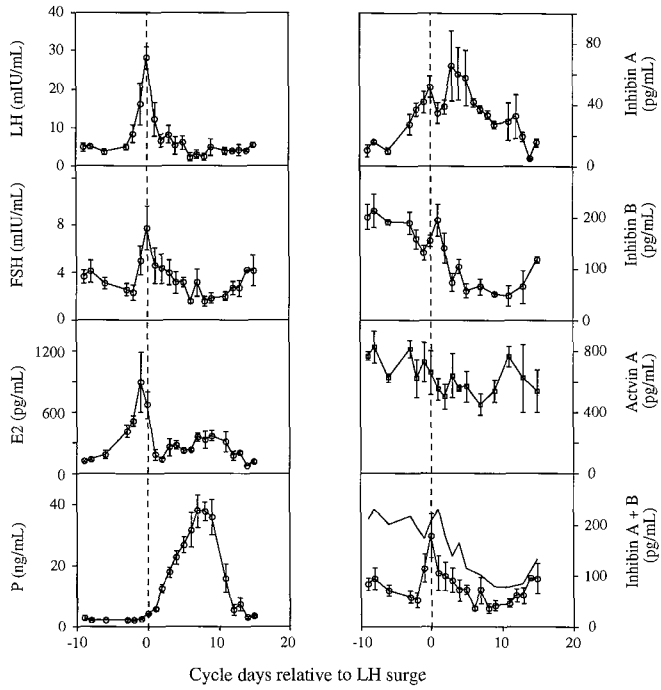

Fig. 3.2 Circulating levels of LH, FSH, E_2, P, inhibin-A, inhibin-B and activin-A during the human menstrual cycle. Data are aligned to the onset of the LH surge (Time 0). Data derived from Muttukrishna *et al.* 2000.

to monitor changes in peripheral levels of these FSH regulatory proteins and assess if they have the potential to have an endocrine role.

Studies employing dimer-specific inhibin assays have shown that levels of circulating inhibin-A are low during the early follicular phase and increase during the late follicular phase, with the highest levels being found during the luteal phase of the cycle (Groome *et al.* 1994, McConnell *et al.* 1996, 1998, Muttukrishna *et al.* 1994, 1998) (Fig. 3.2). Inhibin-B levels on the other hand are high during the early follicular phase, fall during the late follicular phase, transiently increase two days after the gonadotropin surge and then drop to a nadir during the mid-luteal phase (Groome *et al.* 1996)

(Fig. 3.2). In essence, inhibin-A and B levels change in the opposite direction during the early follicular and luteal phases. This raises an interesting question. Does the net peripheral inhibin input change in a meaningful manner to elicit an endocrine role?

Considering the FSH-suppressive effects of both inhibin-A and inhibin-B (Ying 1987, 1988), the sum total of inhibin-A and inhibin-B levels should provide an index of total peripheral inhibin concentration. However, there are three issues that are to be resolved prior to assessing net inhibin contribution by summing inhibin-A and B levels. It should be recognised that this is a highly derived number. For this estimate to be meaningful, we need to first establish that the immunoassays for inhibins A and B predict the respective protein masses correctly. The second issue that requires resolution is if the relative affinity of the inhibin/activin receptors for these two isomers and the biologic potency of these two isomers to suppress human FSH are similar. Both inhibin-A and inhibin-B have similar molecular weights and have been shown to be equipotent in suppressing FSH from rat pituitary cells in culture (Ying 1987, 1988, Robertson *et al.* 1997). Available information also suggests that inhibin-B binds the activin-A receptor with much lower affinity than inhibin-A. In contrast, sheep pituitary cells are relatively insensitive to inhibin-B (Robertson *et al.* 1997). Similar information is not available for the human.

The third issue to be resolved is if the levels of inhibin and the degree of changes observed in peripheral circulation are of sufficient magnitude to elicit an effect at the pituitary level, especially when the pituitary itself is known to produce these regulators. Identification of two binding sites with binding constants of 0.28 and 3.9 nM by Hertan *et al.* (1999) in the ovine pituitary makes this a likely possibility, although it remains to be determined if binding of inhibin to these sites represents an inhibin-specific receptor capable of transducing an intracellular signalling.

Assuming that inhibin-A and inhibin-B assays correctly estimate inhibin mass and that the two are equipotent biologically, it appears total inhibin tone (sum of inhibin-A and inhibin-B is high during the early follicular phase, declines during the pre-surge period, peaks two days after the pre-ovulatory gonadotropin surge and then declines steadily to reach a nadir

during the mid-luteal phase. If this estimate is correct, the direction of changes in total inhibin closely follows changes in FSH. The close tracking of FSH and inhibin raises several questions. Does inhibin have an endocrine role in regulating FSH? Is FSH itself directing changes in inhibin levels? Is there a close feedback regulation between ovarian inhibin and pituitary FSH production? Immunoneutralisation studies have provided perhaps the most compelling evidence to support a role for inhibin in the negative feedback regulation of pituitary FSH secretion in a range of species including the rat (Rivier *et al.* 1986), sheep (Wrathall *et al.* 1990) and cattle (Glencross *et al.* 1992). While these studies establish that inhibin is a negative feedback regulator of FSH, it remains to be determined whether this mediation occurs via neutralisation of pituitary or ovarian production of inhibin because both the ovary and pituitary produce inhibin.

On the contrary, it has been inherently more difficult to determine the reciprocal role of FSH in the regulation of inhibin production because only recently assays which accurately measure dimeric inhibins A and B in serum have been available. Many early reports in the literature which utilised inhibin assays that detected inhibin dimer and inhibin α-subunit alike suggest that FSH can indeed increase peripheral levels of inhibin, but whether this increase is a reflection of increase in biologically active inhibin dimers or an increase in inhibin-α is not known. For example, Fahy *et al.* (1995) reported in pregnant mare serum gonadotropin (PMSG)-treated immature rats that PMSG increases serum levels of both inhibin-A and total inhibin-α. Studies in adult rats show that plasma inhibin-A and FSH concentrations across the whole cycle are negatively correlated whereas no correlation exists between total inhibin-α and FSH (Fahy *et al.* 1995).

The specific role of FSH in short-term regulation of ovarian production of inhibin is also not clear. Follicle-stimulating hormone increases the release of inhibin from isolated human and porcine granulosa cells (Bergh *et al.* 1997, Demura *et al.* 1993). In contrast, FSH treatment has no effect on inhibin production from ovine granulosa cells (Campbell *et al.* 1996). Furthermore, *in vivo* studies in sheep also suggest that ovarian secretion of inhibin is not acutely responsive to FSH (Campbell *et al.* 1991). These data stress the need to further investigate the differential regulation of inhibin-α,

inhibin-A and inhibin-B by FSH. An issue to take into account when considering if a closed-feedback loop exists between FSH and peripheral levels of inhibin is that the stimulatory effect of FSH on inhibin secretion from granulosa cells appears at least in part to be mediated via an increase in transcription (LaPolt *et al.* 1990) and therefore may not lend itself to acute regulation. Similarly the effects of inhibin on FSH also involve changes in FSHβ transcription (McNeilly *et al.* 1995, Farnworth 1995), and hence may take time to manifest at the peripheral level (Padmanabhan *et al.* 1997c).

While changes in peripheral inhibin have the potential to affect FSH and therefore have an endocrine role, circulating levels of total follistatin do not differ significantly during different phases of the menstrual cycle in normally cycling women (Khoury *et al.* 1995, Gilfillan & Robertson 1994, Kettel *et al.* 1996, Wakatsuki *et al.* 1996, McConnell *et al.* 1998) (Fig. 3.3). Measurement of activin-free follistatin (McConnell *et al.* 1998) in circulation shows that only a small portion of circulating follistatin may be in the free

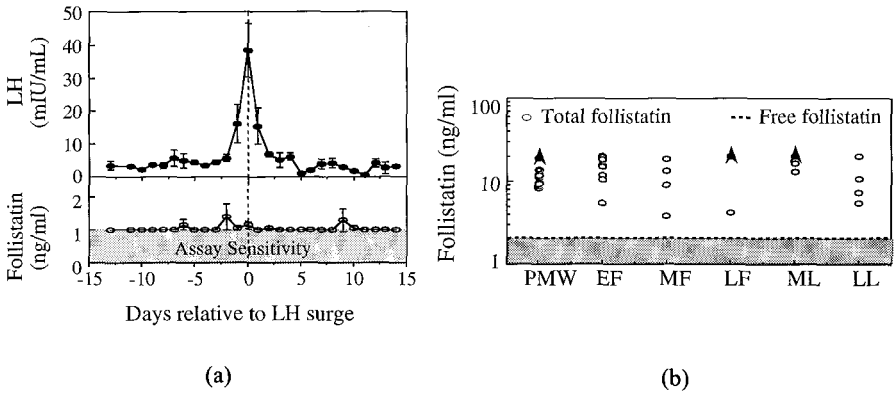

Fig. 3.3 Circulating levels of follistatin during the human menstrual cycle. Left: Shown are patterns of circulating LH and free follistatin (follistatin not bound to activin) during the human menstrual cycle. Data are aligned to the onset of the LH surge (Time 0). Circulating levels of free follistatin were at or below the sensitivity of the assay (1 ng/ml). Right: Shown are patterns of circulating follistatin (total vs. activin-free) during different phases of the human menstrual cycle and in post-menopausal women. Redrawn from McConnell *et al.* 1998. PMW — post-menopausal women; EF — early follicular phase; MF — mid-follicular phase; LF — luteal phase, ML — mid-luteal phase; LL — late luteal phase.

form in normally cycling women (Fig. 3.3). In contrast to low concentrations of free follistatin in the circulation, substantial amounts of activin-free follistatin are found both in the ovary (e.g. follicular fluid) and the pituitary (McConnell *et al.* 1998).

While direct studies are required, presence of free follistatin at multiple sites suggests that free follistatin produced at these sites may be secreted and then complex with activin in the circulation. These considerations are consistent with the premise that the follistatin protein is an unlikely endocrine factor but appears to be an important element in limiting the bioavailability of activin to local sites. Support for the premise that the role of follistatin in circulation is to limit activin availability also comes from studies quantifying free activins A and B in circulation. Using an immunoassay specific for detecting free activins A and B, Woodruff and colleagues found very little biologically active, free activins A or B in peripheral circulation of normally cycling women (Woodruff *et al.* 1994). Chromatographic studies of Muttukrishna *et al.* (1996) provide further support, and show that almost all activin-A in circulation is in a bound state in pregnant women. In direct contrast to these reports, Demura *et al.* (1992), utilising a competitive protein binding assay, estimated much higher levels of circulating "free" activin (~2 ng/ml). The differences between reports in estimating free circulating activin (Demura *et al.* 1992, Woodruff *et al.* 1994) may relate to differences in the ability of these assays to measure various circulating forms of activin (A, B and AB).

Differences between measurements of free follistatin and total follistatin in circulation also suggest that circulating activin should be at far higher concentrations (~8 ng/ml) than levels estimated by the total activin-A assay alone. Circulating concentrations of total activin-A average only 100–200 pg/ml during the human menstrual cycle (Knight *et al.* 1996, Muttukrishna *et al.* 1996) (Fig. 3.2). Circulating levels of total activin-AB during various physiologic states were less than 190 pg/ml (sensitivity of the assay) (Evans *et al.* 1997). Information regarding levels of circulating total activin-B are not available and await validation of assays specific for measuring activin-B. While it is premature and may be erroneous to make quantitative

comparisons across published reports involving different patient populations as well as different assay formats and standards, these estimates bring to the forefront the complicated relationship that exists between follistatin and activin in circulation and establishing if they have endocrine roles. Thus, it is becoming increasingly clear that follistatin and activin may not function as endocrine factors during reproductive cyclicity.

Overall, considering the possible lack of an endocrine role for activin and follistatin, the net peripheral contribution of these FSH regulators, if any, appears to be one of inhibition and mainly dictated by inhibin.

3.3.2 Pituitary Contribution

In addition to peripheral contribution, recent studies have demonstrated yet another level of novel FSH control — autocrine or paracrine regulation within the pituitary (DePaolo *et al.* 1991b, Mather *et al.* 1992, Corrigan *et al.* 1991, Bilezikjian *et al.* 1994). Inhibin, activin and follistatin have been shown to exist in the rat, bovine and human pituitaries (Farnworth *et al.* 1995, Gospodarowicz & Lau 1989, Roberts *et al.* 1989, Mather *et al.* 1992, Bilezikjian *et al.* 1993a, 1993b, Kogawa *et al.* 1991, DePaolo *et al.* 1991b, 1993, Kaiser *et al.* 1992, Halvorson *et al.* 1994, Meunier *et al.* 1988).

Corrigan and co-workers (Corrigan *et al.* 1991) provided the first evidence for local pituitary control of FSH by activin. They demonstrated that activin-B antibody suppresses FSH secretion from cultured rat pituitary cells. Ovine pituitary perifusion studies have provided further support for this premise (Padmanabhan *et al.* 1995a), these studies show that basal FSH secretion increases gradually during perifusion and then stabilises during long-term perifusion. Treatment with follistatin (Padmanabhan *et al.* 1995a) or inhibin (Padmanabhan *et al.* 1995b) prevents this increase or suppresses basal FSH secretion. Because both inhibin and follistatin antagonise activin action and thus inhibit FSH secretion (Carroll *et al.* 1991, Weiss *et al.* 1993, Padmanabhan *et al.* 1995a, 1995b), the regulatory effects of this intra-pituitary loop can be viewed as the net activin availability (the portion of activin that is not opposed by follistatin or inhibin, Fig. 3.4).

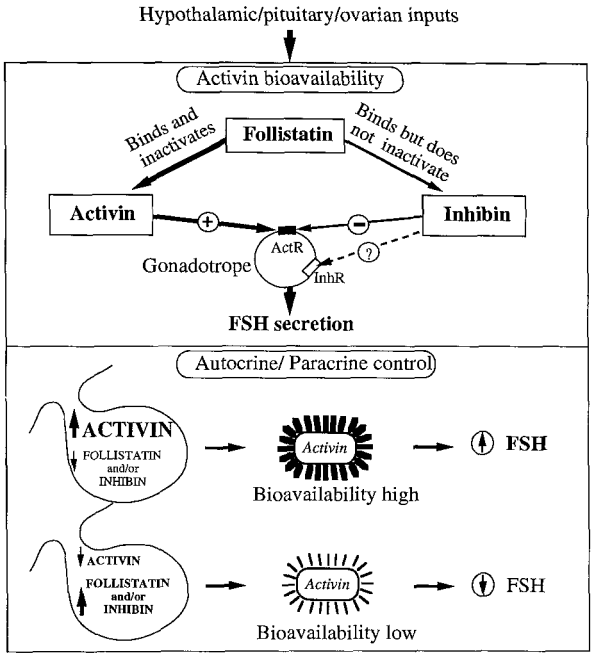

Fig. 3.4 Model for pituitary paracrine/autocrine control showing the interactions of activin, inhibin and follistatin in regulating activin bioavailability and consequent changes in FSH secretion. The model predicts that basal FSH secretion is regulated by activin bioavailability. The relative proportions of follistatin, inhibin and activin dictate the bioavailability of activin. If inhibin and/or follistatin tone is high FSH secretion is low and if inhibin and/or follistatin tone is low, FSH secretion is high.

3.4 Control of Pituitary Activin Bioavailability

How is this local feedback loop regulated? Earlier studies in HPD ewes have shown that FSH secretion is markedly reduced following ablation of neural input for several days (Clarke *et al.* 1983, Hamernik & Nett, 1988) This suggests that the ultimate regulatory control for a major portion of basal FSH release may still reside within the hypothalamus. Studies characterising FSH secretion following administration of follistatin also suggest that the

impetus for setting the FSH regulatory tone can be initiated far in advance of observation of changes in peripheral concentrations of FSH (Padmanabhan *et al.* 1997c). This phenomenon stems partly from the long half-life of this hormone (Ulloa Aguirre *et al.* 1995) and opens the possibility that seemingly unrelated observations may indeed be directly coupled. Regulation of the bioavailability of activin and the consequent change in peripheral FSH secretion may involve multiple intermediary steps (stimulus → changes in FSH regulatory proteins → alteration in activin bioavailability → FSH biosynthesis → FSH secretion), such that coupled events appear unrelated. Therefore, it is entirely possible that ultimate regulation of activin bioavailability may still be mediated by GnRH and may involve several intermediary steps.

3.4.1 GnRH Regulation of Activin Availability

In conjunction with steroids, GnRH appears to differentially regulate mRNA expression of pituitary activin, inhibin and follistatin (Bilezikjian *et al.* 1993b, 1996). For example, the continuous presence of GnRH for two or six hours increases follistatin mRNA levels and decreases mRNA levels of β_B in rat anterior pituitary cells in culture (Bilezikjian *et al.* 1996). However, the effects of GnRH on follistatin and β_B mRNA levels may be dependent on the mode of delivery of GnRH. Indeed, in male rats, regulation of follistatin gene expression by pulsatile GnRH is frequency-dependent, with only fast GnRH pulse frequencies (30 minutes or less) stimulating follistatin gene expression (Kirk *et al.* 1994).

In general, a reciprocal relationship exists between the expression of FSHβ and follistatin mRNAs in response to different patterns of GnRH treatments in perifused, rat pituitary cells (Kirk *et al.* 1994, Besecke *et al.* 1996). Infrequent pulses of GnRH (five-minute pulses for 30 or 60 minutes) stimulate only FSH mRNA expression whereas higher frequency pulses (five-minute pulses for 15 minutes) or continuous GnRH increase follistatin mRNA and protein expression (Besecke *et al.* 1996). Interestingly, decreasing the frequency of GnRH pulses also leads to increased levels of circulating

FSH *in vivo* (Wildt *et al.* 1981). Furthermore, the studies of Besecke *et al.* (1996) showed stimulation of FSHβ mRNA by hourly GnRH pulses can be blocked by follistatin, suggesting that GnRH stimulation of FSHβ mRNA requires endogenous activin. In a similar fashion to the differential regulation of follistatin in response to GnRH pulse frequency, β_B mRNA may also be differentially regulated depending on the pattern in which GnRH is delivered. Although continuous or rapid pulses of GnRH may preferentially stimulate the accumulation of follistatin mRNA, a slower pulse frequency may more effectively stimulate the production of β_B mRNA. Studies of Dalkin *et al.* (1998) have shown that low amplitude GnRH pulses increase activin β_B mRNA expression. This raises the possibility that the amplitude modulation of FSHβ mRNA by GnRH pulse amplitude may in fact be mediated via alteration in activin availability.

3.4.2 Influence of Gonadal Steroids on Activin Availability

Gender-related differences are also apparent in the regulation of pituitary activin, inhibin and follistatin. For example, after gonadectomy, expression of inhibin-α mRNA is unchanged and follistatin mRNA expression increases to a similar degree in both sexes, but β_B is only increased in female rats (Dalkin *et al.* 1998). Replacement with oestradiol prevented the increase in β_B mRNA expression after ovariectomy (Dalkin *et al.* 1998). Furthermore, in male rats, treatment with a GnRH antagonist completely prevented the increase in follistatin mRNA after gonadectomy (Dalkin *et al.* 1998). However, in contrast to male rats, GnRH blockade only partially suppressed follistatin mRNA expression in antagonist-treated females after ovariectomy such that mRNA concentrations were higher than those in intact females (Dalkin *et al.* 1998). Administration of a GnRH antagonist in conjunction with oestradiol did not further reduce mRNA levels of β_B (Dalkin *et al.* 1998). These differences between male and female animals as well as between mRNA species could result from different regulatory responses to GnRH, gonadal steroids, and/or gonadal peptides.

3.4.3 Influence of FSH-Regulatory Proteins on Activin Availability

Activin, inhibin and follistatin also appear to exert regulatory actions on themselves and thus activin bioavailability. In cultured rat pituitary cells, both pituitary-derived and exogenous activin can stimulate follistatin and β_B gene expression (Dalkin *et al.* 1996, Bilezikjian *et al.* 1996). In addition, follistatin can decrease its own mRNA levels but does not appear to have an effect on inhibin-α or β_B (Dalkin *et al.* 1996, Bilezikjian *et al.* 1996). As with many of the other steady state mRNA studies, these data show that the balance between these regulatory elements may be under pituitary paracrine control, but similar studies need to be undertaken at the protein level. The physiological significance of the observed changes in pituitary mRNA levels requires further investigation. The mechanisms that regulate pituitary activin, inhibin and follistatin expression are clearly complex and may involve changes in transcription rates, in mRNA stability and/or degradation, as well as in protein synthesis and turnover rates. All in all, the local production of activin, inhibin and follistatin, and ultimately the bioavailability of activin, represent a potential site where hypothalamic or systemic endocrine signals may be integrated to regulate FSH synthesis and secretion.

3.5 Requirements for Establishing a Local Autocrine/Paracrine Role for the FSH Regulatory Proteins

In the late 1800s, Robert Koch described a set of postulates that must be fulfilled in order to establish a microorganism is responsible for a disease. Likewise, to establish that FSH secretion is under local pituitary autocrine/paracrine control, we need to fulfill three criteria. The first criterion is that these regulators must exist at the pituitary level in a biologically active form. Several studies have provided supportive evidence by documenting that mRNAs for these regulators are present in the pituitary (Roberts *et al.* 1989, Mather *et al.* 1992, Bilezikjian *et al.* 1993a, Kogawa *et al.* 1991, DePaolo *et al.* 1991, 1993, Kaiser *et al.* 1992, Halvorson *et al.* 1994, Meunier *et al.* 1988). Apart from the establishment of follistatin production at the pituitary level (Farnworth *et al.* 1995, Gospodarowicz & Lau, 1995, Besecke

et al. 1996, 1997), such information remains to be documented at the protein level for activin and inhibin.

The second criterion is that activin bioavailability within the pituitary should change in a meaningful manner in relation to changes in FSH secretion: i.e. activin bioavailability should be higher when FSH secretion is high (such as during the primary gonadotrophin surge and secondary FSH surge), and lower when FSH secretion is low (follicular phase). Studies carried out thus far have characterised changes in steady state mRNA levels of the various regulatory proteins during different physiologic states (DePaolo *et al.* 1993, Halvorson *et al.* 1994, Bauer-Dantoin *et al.* 1996, Dalkin *et al.* 1994, 1996). While these mRNA studies support the existence of such reciprocal relationships between FSH regulatory protein mRNAs and FSH, these studies have not been extended to addressing changes in FSH regulatory protein levels. Furthermore, since the signalling cascade of activin is mediated via four different, receptor types (Mathews & Vale, 1991, Attisano *et al.* 1992, Mathews 1994), to get a precise assessment of changes in activin availability, one also needs to assess changes in the different activin receptor sub-types.

The third criterion is that the locally produced FSH regulatory proteins should be involved in the control of FSH secretion, such that imposed changes in local activin bioavailability should lead to corresponding changes in FSH secretion. Barring the initial *in vitro* studies by Corrigan *et al.* (1991), this has not been investigated thoroughly at the protein level. This would require selectively ablating/inhibiting determinants of activin availability in the pituitary and ascertaining if such ablation leads to anticipated changes in FSH secretion. For instance, would ablation of pituitary follistatin lead to an increase in activin bioavailability and a consequent increase in FSH secretion? While several transgenic models (Guo *et al.* 1998, Nishimori & Matzuk, 1996, Matzuk *et al.* 1996) are available to link the various FSH regulators to the control of FSH, these models do not segregate between local (autocrine/paracrine action at the pituitary level) and endocrine (peripheral) involvement. Targeted and localised ablation of the various FSH regulatory proteins at the pituitary is required to establish if these local modulators have a physiological role.

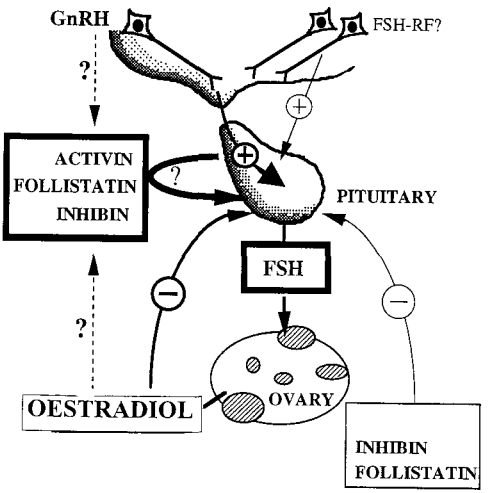

Fig. 3.5 Neuroendocrine control of FSH secretion. Control of FSH is complex, involving multiple regulatory mechanisms. Hypothalamus provides positive input via GnRH and possible FSH-releasing factors. The sum effect the ovary provides appears to be negative and mainly dictated by oestradiol and possibly inhibin. Ovarian-derived activin and follistatin do not possess an endocrine role. A third level of regulation appears to be mediated via changes in FSH regulatory proteins and at the level of the pituitary.

3.6 Key Points and Future Direction

The following key points summarise our current understanding of FSH regulation (Fig. 3.5) and the endocrine and paracrine/autocrine roles of inhibin, activin and follistatin in regulating this key reproductive hormone.

- In light of the pivotal role FSH plays in controlling follicular development, multiple regulatory mechanisms may serve to ensure its proper production and release.
- At one level, positive inputs from the hypothalamus (GnRH and yet to be characterised FSH-RFs) appear to be involved in the control of episodic FSH release.
- At a second level, the ovary provides negative inputs via oestradiol (a major negative feedback hormone of FSH) and inhibin.

- A third level of control appears to be *mediated via factors residing* in the pituitary itself and involves an autocrine/paracrine loop comprised of activin, inhibin and follistatin — three known regulators of FSH secretion.

- The ultimate control of activin availability and the threshold of FSH secretion may still reside within the hypothalamus.

While there is little doubt that large strides have been made in support of a role for inhibin, activin and follistatin in the control of FSH, we are far from gaining a full understanding of the nature of this regulation. Several questions remain unanswered. Do these mediators operate solely in a paracrine/autocrine model? Can we rule out an endocrine contribution all together? What regulates this local regulatory loop? Do these local regulators have a physiological role? To fully answer these questions we require: (1) specific assays that detect the bound and free components of each of the regulators contributing to this loop, (2) pure forms of each of these FSH regulatory proteins in sufficient amounts to carry out *in vivo* studies, and (3) approaches to target ablation of the components of the local loop at local sites and study the impact on FSH during various physiological states.

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CHAPTER 4

REGULATION OF PRODUCTION AND INTRAOVARIAN ROLES OF INHIBIN, ACTIVIN AND FOLLISTATIN

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Inhibin, activin and follistatin were initially isolated from follicular fluid based on their endocrine action on pituitary follicle stimulating hormone (FSH) secretion. FSH also stimulates the production of these proteins in the ovary and forms a negative feedback loop with inhibin. In recent years, research has shown that inhibin, activin and follistatin also have autocrine and paracrine functions within the ovary, modulating cell proliferation, gonadotrophin responsiveness, steroidogenesis and oocyte maturation. This chapter reviews the factors that control ovarian production of these proteins and their local actions within the ovary.

4.1 Introduction

It is estimated that a human ovarian follicle takes the equivalent of approximately three menstrual cycles (85 days) to grow from the early pre-antral stage (150 μm) to ovulatory size (20 mm). Within the reproductive life-span of a normal woman, it is unlikely that more than 500 of the one to two million primordial follicles her ovaries contain at birth will complete

this complex developmental pathway; the vast majority (>99.95%) will undergo atresia long before reaching ovulatory potential. Profound alterations in cytoproliferation and differentiative function of somatic cells (expression of receptors for hormones, growth factors; expression of steroidogenic enzymes, peptide hormones) and changes in oocyte morphology and biochemistry accompany this dramatic enlargement of the follicular compartment. The presumptive regulatory signals that bring about the ordered recruitment of quiescent primordial follicles into the growing pool have not been determined but follicles up to the small antral stage (~2 mm) are thought to be insensitive to FSH. Beyond ~2 mm follicles acquire functional FSH receptors and, from this subset of FSH responsive follicles, some are selected to continue along their developmental pathway under the trophic influence of both FSH and luteinising hormone (LH) (Gougeon 1996).

In recent years several growth factors have been identified at the ovarian level and implicated as co-regulators of folliculogenesis along with gonadotrophins of pituitary origin. These include IGF I and II, EGF, TGFα, FGF, TGFβ, GDF-9, inhibin, activin and follistatin (Adashi & Rohan 1992, Giudice *et al.* 1993, McGrath *et al.* 1995). Intraovarian expression of a particular growth factor, or its receptor, may itself be gonadotrophin-dependent, in which case it may act as a local mediator of gonadotrophin action. Alternatively, a growth factor may exert regulatory actions by modulating the responsiveness of follicular cells to gonadotrophins. Growth factors that amplify FSH responsiveness would most likely favour follicle survival while those that attenuate FSH responsiveness would induce atresia. Findlay (1993) suggested a number of criteria that should be fulfilled for a substance to be deemed an autocrine or paracrine factor:

- Local production of the factor must be demonstrated (e.g. expression of mRNA, immunochemical localisation within cells, net synthesis *in vivo*).

- The production of the factor must be shown to be regulated (e.g. by peripheral or local hormones).

- The factor should elicit a demonstrable cellular response, either on the same cell type that secreted it (autocrine action) or on neighbouring cells (paracrine action).
- The action of the factor should be regulated at a local level (e.g. by binding protein, formation from a pro-hormone, degradation).
- The local action of the proposed autocrine/paracrine factor should be demonstrated *in vivo*.

The above criteria serve as useful guidelines when considering current evidence supporting autocrine/paracrine roles of inhibin, activin and follistatin in the human ovary; with the exception of the final criterion, they have largely been fulfilled. While this article gives emphasis to studies of human

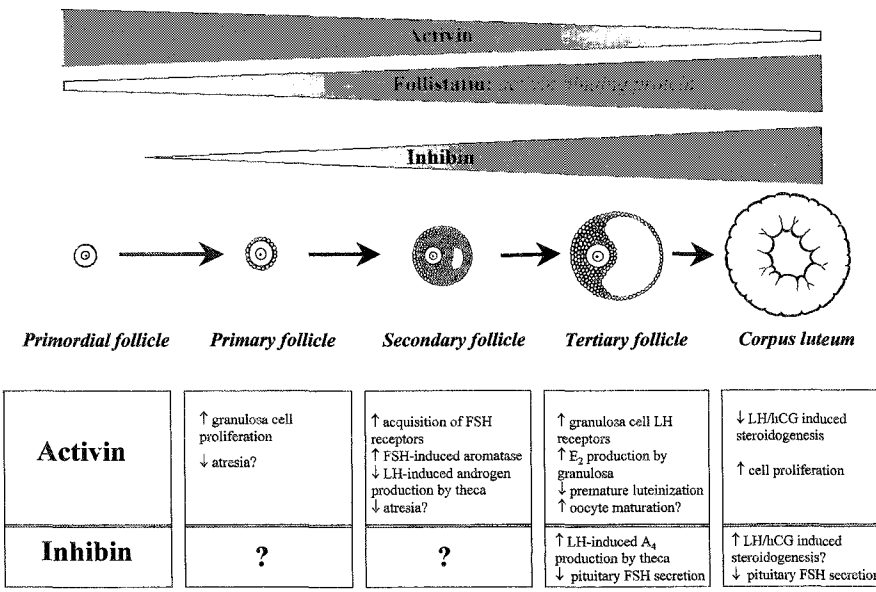

Fig. 4.1 Schematic diagram linking likely developmentally-related changes in granulosa cell expression of inhibin, activin and follistatin with their putative intraovarian and endocrine actions.

and non-human primates, data from non-primate species are also referenced, particularly in areas where primate data are lacking.

Inhibin, activin and follistatin were initially characterised as ovarian endocrine factors based on their ability to regulate pituitary FSH release (Ying 1988). *In vitro* studies on isolated granulosa cells, theca cells and oocytes from several species including human and non-human primates have provided compelling evidence that these proteins exert local autocrine/paracrine actions to modulate follicular growth, steroidogenesis, oocyte maturation, ovulation and corpus luteum function (Fig. 1).

4.2 Expression of Inhibin, Activin and Follistatin and their Receptors in the Primate Ovary

Human follicular fluid has been shown to contain inhibins A and B, three isoforms of activin (A, AB and B), several size variants of follistatin and several forms of monomeric inhibin α-subunit (Knight *et al.* 1989, Knight *et al.* 1996, Evans *et al.* 1997, Schneyer *et al.* 1997). The distribution of mRNAs encoding the α, β_A and β_B inhibin/activin subunits and follistatin in human and primate ovarian follicles and corpora lutea has been examined by *in situ* hybridisation (Schwall *et al.* 1990, Fraser *et al.* 1993, Roberts *et al.* 1994, Tuuri *et al.* 1996, Sidis *et al.* 1998). Likewise, the localisation of the respective translated proteins has been determined using immunohistochemistry (Yamoto *et al.* 1991, 1992, Rabinovici *et al.* 1992, Roberts *et al.* 1993). Follicular granulosa cells and granulosa-lutein cells of the corpus luteum are the principle sites of expression of inhibin/activin α, β_A and β_B subunits and follistatin, although at certain stages of follicle development, theca cells have also been shown to express some of these proteins, particularly α-subunit. In general, pre-antral and small antral follicles express β_A and β_B subunits but little α-subunit or follistatin. Large healthy follicles also express β_A and β_B but with increasing amounts of α-subunit and follistatin; expression of each protein is diminished in large atretic follicles. Corpus luteum express α, β_A and follistatin but little β_B.

Activin receptors have been identified on granulosa cells, theca cells and oocytes consistent with local actions on these cell types (Woodruff *et al.* 1993, Eramaa *et al.* 1995, Peng *et al.* 1996, Izadyar *et al.* 1998, Sidis *et al.* 1998). Despite intensive effort, specific inhibin receptors have not yet been cloned and characterised, although recent evidence from mouse and sheep supports the existence of such a receptor (Draper *et al.* 1998, Hertan *et al.* 1999). At least some of the biological actions of inhibin may be mediated at the level of the activin receptor since inhibin can bind to cloned activin type II receptors, albeit with a much lower affinity than activin, and prevent activin-induced heteromerisation of type I and II receptors, a prerequisite for activation of the intracellular signal transduction cascade (Mathews 1994, Lebrun *et al.* 1997).

Follistatin, a high affinity activin-binding protein, can neutralise the effects of activin by blocking its interaction with specific receptors on target cells. Follistatin also binds to inhibin but with much lower affinity; this binding does not appear to neutralise inhibin bioactivity. With regard to activin-binding, the situation is more complex because multiple isoforms of follistatin exist which may subserve different functions (Nakamura *et al.* 1997, Schneyer *et al.* 1997, Patel 1998). The shorter truncated isoforms associate with cell surface heparin sulphate proteoglycans and may serve either to entrap locally produced activins near the cell surface and/or prevent activins from more distant sites reaching receptors on the cell surface. There are conflicting theories, which have yet to be reconciled, as to the consequences of activin-binding to membrane-anchored (shorter) follistatin isoforms: the process may (i) neutralise activin by blocking its interaction with its type I or II receptor(s); (ii) reduce activin action by promoting endocytic internalisation leading to lysosomal degradation of activin by the cell; (iii) increase activin action by facilitating its presentation to activin receptor(s). The longer isoforms of follistatin have much reduced affinity for cell surface proteoglycans and it is envisaged that they serve to bind and neutralise activins in extracellular fluid and in the peripheral circulation, thus limiting their action at more distant sites in the body.

4.3 Factors Regulating Granulosa Cell Production of Inhibin, Activin and Follistatin

4.3.1 Gonadotrophins

Administration of FSH increases circulating levels of both inhibins A and B in women (Muttukrishna *et al.* 1994, Lockwood *et al.* 1996). These findings are consistent with *in vitro* studies on isolated granulosa or granulosa-lutein cells from human and non-human primates which also show that the stimulatory effects of FSH on inhibin secretion and expression of α and β subunits can be mimicked by agents which raise intracellular levels of cAMP (Hillier *et al.* 1989, 1991a, Eramaa *et al.* 1994, Tuuri *et al.* 1996, Bergh *et al.* 1997). The promotor regions of α-, β_A- and β_B-subunit genes contain putative regulatory elements for cAMP consistent with the involvement of a protein kinase A-dependent signalling pathway (Mayo 1994, Mason *et al.* 1989, Eramaa *et al.* 1994, Tuuri *et al.* 1996).

Luteinising hormone human chorionic gonadotrophin (LH/hCG) have also been shown to stimulate granulosa cell inhibin production in a cAMP-dependent manner but only with cells from mature pre-ovulatory follicles (i.e. human granulosa-lutein cells) that have acquired LH receptors under the influence of FSH (Tsonis *et al.* 1987, Hillier *et al.* 1989, Eramaa *et al.* 1994, 1995). In non-primates granulosa cell expression of inhibin α and β subunits falls dramatically after the pre-ovulatory gonadotropin surge and corpora lutea express little or no inhibin (Knight 1996). In contrast, in human and non-human primates, luteal tissue *in vivo* and both granulosa-lutein and dispersed luteal cells *in vitro* continue to express α and β_A subunits (with reduced β_B expression) and to secrete inhibin-A in an LH/hCG-responsive manner (Fraser *et al.* 1992, 1995, Schwall *et al.* 1990, Yamoto *et al.* 1991, Eramaa *et al.* 1994, Muttukrishna *et al.* 1997). Indeed, during the mid-luteal phase of the human menstrual cycle, circulating levels of inhibin-A are maximal (Groome *et al.* 1994, Muttukrishna *et al.* 1994) while inhibin-B levels are at their lowest level (Groome *et al.* 1996). In monkeys, suppression of LH secretion during the mid-luteal phase by administration of a gonadotrophin-releasing hormone (GnRH) antagonist promotes a

reduction in plasma inhibin and progesterone, which can be prevented by giving exogenous LH/hCG to "rescue" the corpus luteum (Smith & Fraser 1991, Webley *et al.* 1992).

There have been few studies on the regulation of activin production by ovarian follicles of primates, largely due to the unavailability of specific assays for measuring the different activin isoforms. In women undergoing *in vitro* fertilisation (IVF), neither pituitary down-regulation with GnRH analogue (to arrest follicle development), nor subsequent administration of FSH (to promote multiple follicular development), affected plasma activin-A levels (Lockwood *et al.* 1996). Similarly circulating activin-A levels show little variation during the human menstrual cycle (Muttukrishna *et al.* 1996). Unfortunately, *in situ* hybridisation analysis of inhibin/activin subunit mRNA expression and subunit immunolocalisation studies do not reveal the extent to which translated subunit gene products are assembled to form functional inhibin or activin dimers. For instance, evidence from human and monkey ovary (Schwall *et al.* 1990, Yamoto *et al.* 1992) that granulosa cells of small follicles express β_A or β_B subunits but not α-subunit implies that immature follicles synthesise activins but lack the capacity to synthesise inhibins. In contrast, more mature follicles express α, β_A and β_B subunits and therefore acquire the potential to synthesise inhibins A and B.

Circulating levels of follistatin increase in women undergoing FSH-induced multiple follicular development (Sugawara *et al.* 1990), although levels are relatively uniform throughout the human menstrual cycle (Evans *et al.* 1998). Expression of follistatin mRNA and protein in human granulosa-lutein cells is strongly stimulated by both FSH (Tuuri & Ritvos 1995) and hCG (Tuuri *et al.* 1994).

4.3.2 Local Regulators

Several locally-produced factors have been shown to stimulate basal and/or gonadotrophin-induced inhibin/activin subunit gene expression or ir-inhibin secretion in primate granulosa cells. These include androgens (Tsonis *et al.* 1987, Hillier *et al.* 1991a), oestradiol (Hillier *et al.* 1989), TGFβ (Eramaa & Ritvos 1996), activin-A (Eramaa *et al.* 1995) and prostaglandin E_2

(Brannian *et al.* 1992a, Tuuri & Ritvos 1995). Interestingly, both activin-A (Eramaa *et al.* 1995) and TGFβ (Eramaa & Ritvos 1996) selectively induced expression of β_B mRNA in human granulosa-lutein cells, without increasing α or β_A expression. Moreover, the effect of activin-A was blocked by both follistatin and hCG. It is notable that this selective effect of activin-A to upregulate β_B-subunit expression in human cells differs from findings in rat granulosa cells (LaPolt *et al.* 1989), in which activin-A induces expression of α and β_A mRNAs. Eramaa *et al.* (1995) also showed that activin-A reduced hCG-induced expression of both inhibin-α and P450scc mRNA in human granulosa-lutein cells, consistent with activin having a negative regulatory action in the corpus luteum to reduce inhibin and progesterone production.

Studies in non-primates have shown that IGF-1 can enhance (Carson *et al.* 1989, Michel *et al.* 1991), while EGF/TGFα (Franchimont *et al.* 1986, Zhiwen *et al.* 1987) and FGF (LaPolt *et al.* 1990) can suppress granulosa cell inhibin production. To our knowledge, comparable studies in primates have not been reported. In the absence of information on the synthesis and secretion of assembled inhibin/activin dimers, the interpretation of many of the above findings based on analysis of subunit mRNA expression alone remains difficult.

Local factors that have been shown to stimulate follistatin expression in human granulosa-lutein cells include EGF and prostaglandin E_2 (Tuuri & Ritvos 1995). In contrast prostaglandin $F_2\alpha$, TGFβ and activin-A had no effect in this study. The luteotrophic action of prostaglandin E_2 could, in part, be explained by its ability to upregulate follistatin expression since this would negate the anti-steroidogenic effect of activin-A referred to above.

4.4 Intrafollicular Actions of Inhibin, Activin and Follistatin

4.4.1 Autocrine/Paracrine Modulation of Granulosa Cell Function

In vitro studies support a local intrafollicular role for activin-promoting granulosa cell proliferation and differentiation. Dose-dependent proliferation of human granulosa-lutein cells in response to activin-A was first reported

by Rabinovici *et al.* (1990). Similar findings have been reported for rat granulosa cells from both small and large follicles (Miro *et al.* 1995, Miro & Hillier 1996, Li *et al.* 1995). In contrast, inhibin has been shown to inhibit ovarian cell proliferation in mice (Matzuk *et al.* 1992).

Although the regulatory mechanisms governing the early stages of follicle development are largely unknown, acquisition of granulosa cell responsiveness to FSH is considered a key event. The observation that activin can promote FSH receptor expression on undifferentiated rat granulosa cells (Hasegawa *et al.* 1988, Xiao *et al.* 1992) may be of particular significance since this could explain how a late pre-antral/early antral follicle progresses from a gonadotrophin-independent stage to a gonadotrophin-dependent stage of development. However, these findings have not yet been confirmed for human or non-human primate granulosa cells.

Once granulosa cells have acquired FSH receptors, their further growth and differentiation would be mainly driven by FSH (and later LH) acting in synergy with activin, but also modulated by follistatin and other locally produced factors (i.e. steroids, insulin-like growth factors, IGF-binding proteins etc.). This putative action of activin to promote FSH receptor expression would operate most successfully in a "low follistatin" environment and there is some evidence that undifferentiated rat granulosa cells express relatively little follistatin in comparison with cells from more developmentally advanced follicles (Nakatani *et al.* 1991, Shimasaki *et al.* 1989). Indirect evidence from *in situ* hybridisation studies on primate ovaries suggests that at early stages of development, follicles preferentially synthesise activin rather than inhibin (Schwall *et al.* 1990, Yamoto *et al.* 1992). However, analysis of the capacity of human follicles at different stages of development to produce each form of inhibin/activin dimer (activins A, AB and BB and inhibins A and B) and follistatin protein will ultimately be required to confirm that there is a functional excess of activin over inhibin and follistatin in early follicle stages.

In vitro studies in a range of species including primates support a role for activin in the regulation of granulosa cell steroidogenesis, although the nature of this involvement appears to differ according to the stage of follicular development. The majority of human studies have involved cultures of granulosa-lutein cells obtained at IVF pick-up. In these developmentally

advanced cells, activin exerts an anti-steroidogenic action, inhibiting basal and gonadotropin-stimulated progesterone secretion, aromatase activity and oestradiol production (Cataldo *et al.* 1994, Rabinovici *et al.* 1992). Eramaa *et al.* (1995) showed that activin specifically inhibits hCG/LH induced P450scc mRNA expression, a finding that corroborates the inhibitory effect on progesterone secretion observed. In contrast, Hillier & Miro (1993) studied non-luteinised granulosa cells from both immature and pre-ovulatory non-human primate follicles, and found that activin enhanced basal and gonadotropin-stimulated aromatase activity. This finding is consistent with other studies of rat and bovine granulosa cells showing that activin can enhance aromatase activity and oestradiol production, whilst inhibiting progesterone secretion (Hutchinson *et al.* 1987, Miro *et al.* 1991, Xiao *et al.* 1990, Shukovski *et al.* 1990, Shukovski *et al.* 1991). This action implies that intrafollicular activin may have a role in delaying the onset of luteinisation.

Follistatin reverses the effect of activin on progesterone secretion by human granulosa-lutein cells (Cataldo *et al.* 1994), whilst having no effect in the absence of activin. Rat studies have shown that follistatin is also able to suppress aromatase activity and inhibin production and increase progesterone secretion (Xiao *et al.* 1990, Xiao & Findlay 1991). This suggests that follistatin can promote follicular atresia (associated with decreased aromatase activity and inhibin production and increased progesterone) or luteinisation, depending on the developmental stage reached.

An early report (Ying *et al.* 1986) that inhibin can suppress FSH-induced aromatase activity in cultured rat granulosa cells, was not substantiated by later studies in rat (Hutchinson *et al.* 1987, Sugino *et al.* 1988) or human (Rabinovici *et al.* 1994). However, in a study involving marmoset granulosa cells (Miro & Hillier 1992), high concentrations of inhibin (100 ng/ml) induced a slight decrease in FSH-induced aromatase activity in granulosa cells from immature, but not mature, follicles. Thus, the present consensus is against inhibin having a significant autocrine/paracrine action to suppress granulosa cell steroidogenesis.

Granulosa cells synthesise an excess of inhibin α-subunit over β_A/β_B subunits (Ying *et al.* 1988, Knight 1996). Several forms of α-subunit monomer have been identified in follicular fluid including full-length α precursor and a number of post-translational cleavage products. None of the free α forms

isolated so far possesses classical inhibin-like bioactivity (ability to suppress pituitary FSH secretion), but there is evidence that the full-length α precursor can compete with FSH for binding to its receptor and so diminish the action of FSH on granulosa cells (Schneyer *et al.* 1991). Thus, the intrafollicular concentration of inhibin α-subunit precursor could be another factor in determining whether a follicle achieves maximum responsiveness to FSH.

Although there is as yet no evidence to implicate its involvement in the human ovary, evidence in sheep suggests that another post-translational product of the α-subunit precursor termed the α_N fragment may subserve a local intrafollicular role to facilitate ovulation. Immunisation of ewes against α_N reduced litter size and disrupted ovulation as indicated by a reduced number of eggs in the oviducts and a greater incidence of luteinised or unruptured follicles in α_N-immunised ewes (Findlay *et al.* 1994). Further work is required to establish the relevence of this observation to the ovulatory mechanism in primates.

4.4.2 Modulation of Androgen Production by Theca Cells

A characteristic feature of a dominant pre-ovulatory follicle is its capacity to synthesise and secrete much greater amounts of oestrogen than its subordinates. This requires a high P450arom activity in granulosa cells and an adequate supply of P450arom substrate (androgen). Since granulosa cells lack P450c17 and are unable to synthesise their own androgen, their ability to synthesise oestrogen is dependent on an adequate supply of androgens from the surrounding theca cells. *In vitro* studies on human (Hillier *et al.* 1991b) rat (Hsueh *et al.* 1987) and bovine (Wrathall & Knight 1995) theca cells have shown that inhibin (a product of gonadotrophin-responsive, oestrogen-active granulosa cells) can enhance LH-induced androgen production. Incubation of whole rat follicles (Smyth *et al.* 1993) or bovine follicle wall segments (J.H.M. Wrathall & P.G. Knight, unpublished observations) with neutralising antibodies to inhibin reduced androgen secretion, consistent with a positive action of endogenous inhibin on thecal androgen production. This could represent an important intrafollicular positive feedback mechanism for ensuring that the pre-ovulatory follicle maintains an adequate supply of substrate for oestrogen synthesis.

Treatment of human, rat and bovine theca cells with activin reduces LH-induced androgen production and opposes the action of inhibin (Hillier *et al.* 1991c, Hsueh *et al.* 1987, Wrathall & Knight 1995). In bovine theca cells, oestradiol itself enhances thecal androgen production (Wrathall & Knight 1995) and this effect was also reduced by activin. Moreover, the inhibitory effects of activin on both LH- and oestradiol-induced androgen secretion were reversed by follistatin, consistent with its role as an activin-binding protein. However, follistatin did not block the stimulatory effect of inhibin on androgen secretion (Wrathall & Knight 1995), despite evidence that it can bind to both activin and inhibin through their common β-subunit (Shimonaka *et al.* 1991). Given the concensus of opinion that theca cells synthesise little or no inhibin, activin or follistatin, collectively these *in vitro* findings suggest that granulosa cell-derived inhibin and activin have mutually opposing paracrine actions to modulate thecal androgen synthesis. The effect of activin (but not inhibin) in this regard probably depends on the local concentration of follistatin.

It has been proposed (Hillier 1991) that in immature follicles which synthesise very little oestrogen, thecal androgen synthesis is also minimal due to a relative excess of activin over inhibin and follistatin (i.e. high "activin tone", low "inhibin tone"). However, as a dominant follicle approa-ches pre-ovulatory status, increasing granulosa cell expression of inhibin and follistatin, perhaps accompanied by decreasing expression of activin (i.e. low "activin tone, high "inhibin tone") upregulate thecal androgen secretion and thereby ensure that the granulosa cells receive an adequate supply of P450arom substrate to match its increasing demand for oestradiol synthesis (Fig. 2). This hypothesis is supported by anatomical evidence of developmental changes in inhibin/activin subunit and follistatin expression during folliculo-genesis (see Sec. 4.2) and by observed changes in intra-follicular concentrations of inhibins and activin-A during follicular develop-ment (Magoffin & Jakimiuk 1998). This study showed that concentrations of activin-A in follicular fluid were similar in individual human follicles ranging from 5–20 mm in diameter whereas inhibins A and B levels increased markedly with follicular size. Unfortunately, intrafollicular follistatin concen-trations were not measured in this study so it was not possible to assess the relative amount of "free" (presumably bioavailable) activin present at different stages.

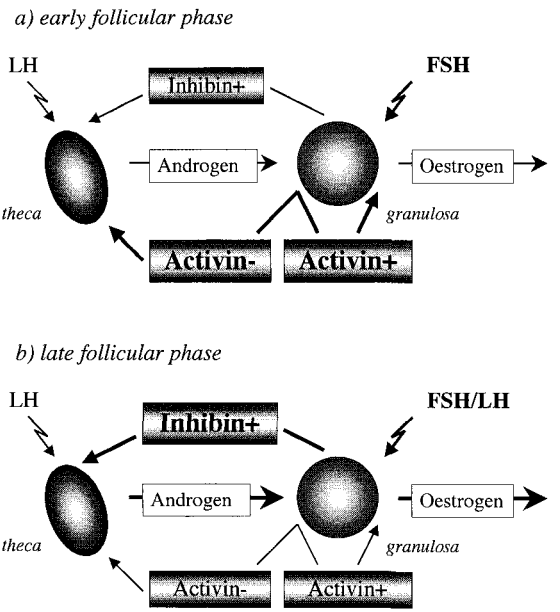

Fig. 4.2 Hypothetical regulatory functions of inhibin and activin in the control of follicular oestrogen synthesis as proposed by Hillier (1991). (a) Early follicular phase: Granulosa cells of immature follicles express high levels of activin but lower levels of its binding protein — follistatin. "Free" activin enhances the inductive action of FSH on aromatase activity, simultaneously suppressing thecal androgen synthesis at a time when oestrogen synthesis is still minimal. (b) Late follicular phase: Granulosa cells of the pre-ovulatory follicle respond to stimulation by FSH and LH with increased production of inhibin, paralleling the pre-ovulatory increase in aromatase activity. Inhibin acts locally to promote LH-stimulated androgen synthesis in the theca interna. As inhibin production increases (possibly in association with reduced production of activin and increased production of follistatin), a positive feedback loop is created, through which thecal androgen synthesis is amplified to sustain oestrogen synthesis in the granulosa cell layer (redrawn from Hillier 1991).

4.4.3 Paracrine Actions to Modulate Oocyte Maturation

Throughout follicle development, oocytes and their surrounding granulosa cells communicate by way of both gap junctions and paracrine signalling factors (Eppig *et al.* 1997). In antral follicles, the oocyte is most heavily reliant on the surrounding cumulus granulosa cells. These differ phenotypically

from the more distant mural granulosa cells, presumably reflecting their proximity to the oocyte and the fact that mural granulosa cells encounter higher levels of systemic (e.g. gonadotrophins) or theca-derived (androgens, growth factors) factors. For example, cumulus cells have greater proliferative capacity, have fewer LH receptors, produce less progesterone but express more inhibin/activin subunits than mural granulosa cells (Channing *et al.* 1981, Roberts *et al.* 1993). Oocyte-secreted factors that are indispensible from a very early stage of follicle development include zona pellucida glycoproteins and GDF-9 (McGrath *et al.* 1995). At later stages of folliculogenesis, factors secreted by the oocyte may prevent premature luteinisation, stimulate hyaluronic acid synthesis by cumulus cells and facilitate cumulus expansion in response to the gonadotrophin surge (Salustri *et al.* 1993). Conversely, factors produced by somatic cells (theca, mural granulosa and cumulus granulosa) are necessary for ordered cytoplasmic and nuclear maturation of the oocyte and for maintaining a state of meiotic arrest until either a pre-ovulatory LH surge occurs or the oocyte is removed from its follicular environment for terminal maturation *in vitro*.

A recent *in situ* hybridisation study of isolated human cumulus-oocyte complexes (Sidis *et al.* 1998) confirmed abundant expression of inhibin/activin α-, β_A- and β_B-subunit mRNAs and follistatin mRNA in cumulus cells (Roberts *et al.* 1993). In contrast, neither β_A nor β_B message were detectable in oocytes, suggesting they lack the capacity to synthesise inhibin/activin dimers. However, all four activin receptor subtypes (IA, IB, IIA and IIB) were detected in oocytes and evidence of differential expression of activin receptors during *in vitro* oocyte maturation was obtained (Sidis *et al.* 1998). These findings support evidence from functional studies in several species that cumulus-derived activin can influence nuclear and cytoplasmic maturation of oocytes. For instance, activin has been reported to accelerate *in vitro* meiotic maturation of oocytes in monkey (Alak *et al.* 1996), rat (Sadatsuki *et al.* 1993) and human (Alak *et al.* 1998), an effect which was inhibited by follistatin. Similarly, Silva & Knight (1998) showed that incubation of denuded or cumulus-enclosed bovine oocytes with activin-A had no effect on cleavage rate after IVF but increased their developmental competence to form blastocysts. Conversely, follistatin reduced oocyte developmental competence and neutralised the effect of both endogenous and exogenous activin.

Evidence implicating inhibin as a potential modulator of oocyte maturation is less consistent although it was reported that inhibin can suppress the spontaneous maturation division of cumulus-enclosed and denuded oocytes from immature rats (O *et al.* 1989). In conflict with other reports, however, activin had no effect in this study. In a recent study on bovine cumulus-enclosed oocytes (Silva & Knight 1999), free inhibin α-subunit, but not inhibin-A, was shown to reduce oocyte developmental competence. Addition of antibodies against α-subunit enhanced oocyte developmental competence consistent with an inhibitory role of endogenous inhibin α-subunit, which is produced in large amounts by cumulus cells. While these *in vitro* findings suggest ways of improving the outcome of assisted reproduction techniques that depend on *in vitro* manipulation of oocytes, further work is required to evaluate their physiological significance.

4.5 Autocrine/Paracrine Actions in Corpus Luteum

Expression of inhibin/activin α and β_A subunits and follistatin is maintained after ovulation in human and non-human primates (Yamoto *et al.* 1991, Roberts *et al.* 1993, Rabinovici *et al.* 1994), being confined to granulosa-lutein cells but not theca-interstitial cells. These observations support possible local actions of inhibin and related molecules in the regulation of luteal function. In this regard, activin has been shown to promote proliferation of cultured human granulosa-lutein cells and decrease their basal and hCG-induced output of progesterone (Rabinovici *et al.* 1990, Li *et al.* 1992, Di Simone *et al.* 1994), an effect which is blocked by follistatin (Cataldo *et al.* 1994). Activin also suppresses progesterone secretion by monkey luteal cells in culture (Brannian *et al.* 1992b). Given that follistatin production is positively regulated by hCG in granulosa-lutein cells (Tuuri *et al.* 1994), follistatin could be an important component of the gonadotrophin-dependent luteal support mechanism.

In contrast to activin-A, inhibin-A had no effect on proliferation or progesterone output of human granulosa-lutein cells either in the presence or absence of activin-A (Rabinovici *et al.* 1994). However, a positive role for endogenous inhibin (or more likely its free α-subunit) in maintaining

luteal progesterone output is suggested by the observation that addition of α-inhibin antibody to human granulosa cells (Webley *et al.* 1994a) or marmoset luteal cells (Webley *et al.* 1994b) reduced hCG-induced progesterone secretion.

4.6 Summary

In this chapter, we have reviewed current evidence implicating inhibin, activin and follistatin as intraovarian regulatory molecules with particular reference to human and non-human primates. Pertinent observations from other species have also been referenced where there is a paucity of information on primates.

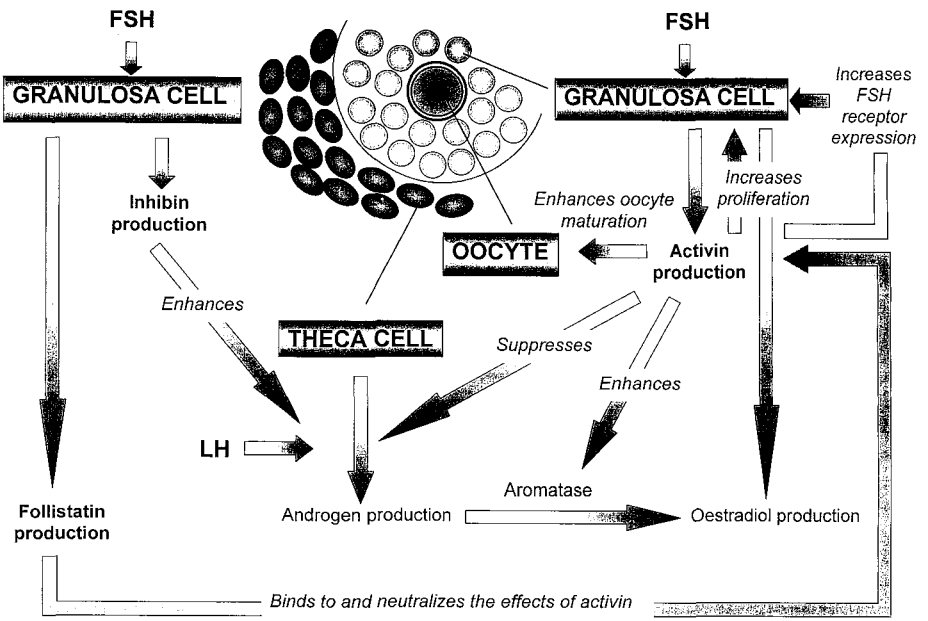

Fig. 4.3 Schematic representation of an ovarian follicle summarising putative autocrine/paracrine actions of granulosa cell-derived inhibin, activin and follistatin on granulosa cells, theca cells and oocytes.

The differential pattern of expression of inhibin/activin subunits and follistatin during follicle development and their differential regulation by systemic and locally-produced factors reinforces evidence from functional studies, mostly *in vitro*, that these proteins subserve physiologically important roles in folliculogenesis. These putative roles are summarised below and in Fig. 3:

Activin:

- Promote granulosa cell proliferation.
- Upregulate FSH receptor expression by granulosa cells.
- Enhance aromatase activity and oestradiol production.
- Increase production of inhibin and follistatin.
- Upregulate LH receptor expression by granulosa cells.
- Inhibit basal and LH/hCG-induced progesterone production by granulosa lutein cells and luteal cells.
- Suppress basal and LH-induced androgen production by theca cells.
- Enhance developmental competence of oocyte.

Follistatin:

- Bind to activin with high affinity.
- Capable of neutralising most of the effects of activin listed above.
- May prevent activin from reaching and/or acting on more distant tissues.

Inhibin:

- Enhance LH-induced androgen production by theca cells.
- No effect on granulosa cell proliferation or steroidogenesis.
- May enhance LH/hCG-induced progesterone production by luteal cells.

Monomeric inhibin α subunit:

- May reduce interaction of FSH with its receptor.
- May have negative effect on oocyte developmental competence.

While there is considerable evidence for differential expression of the various inhibin/activin isoforms during follicle development, virtually all functional studies to date on the actions of exogenous inhibin and activin on follicle cell function have used inhibin-A and activin-A — these two being most readily available to investigators in recombinant form. The possibility that

other inhibin/activin isoforms (including larger forms with amino-terminally extended subunits) have quite different activities cannot be excluded on the basis of present knowledge.

With respect to *in vitro* experiments involving human granulosa cells, it should be pointed out that in most studies, the cells used were obtained from women undergoing gonadotrophin-induced multiple follicular development for IVF. While acknowledging that such granulosa-lutein cells from developmentally advanced follicles are the most readily available form of human material for *in vitro* study, in terms of their production of and responsiveness to inhibin, activin and follistatin, they may differ markedly from granulosa cells of non-stimulated follicles at less advanced stages of development. Thus, there is a need for more information on the functional properties of human granulosa cells from undifferentiated and partially differentiated follicles.

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CHAPTER 5

INHIBIN, ACTIVIN AND FOLLISTATIN IN HUMAN PREGNANCY

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5.1 Introduction

Inhibin, activin and follistatin are members of the TGF-β superfamily. Although these proteins were initially identified and characterised from the gonads, they are widely distributed in other tissues. High circulating levels of dimeric inhibin-A, activin-A and follistatin have been documented throughout pregnancy. Several studies have also identified inhibin/activin subunit mRNAs (α and β) and α- and β-subunit proteins in the placental membranes. Inhibin and activin act within the human placenta through a cell-to-cell communication (paracrine) or within the same cells (autocrine), locally modulating placental hormone production, cell-mediated immune function, cell growth and differentiation of the placenta and the embryo. In addition, placental inhibin-A and activin-A also enter the maternal circulation and may have an endocrine effect during pregnancy. Research on inhibin and related proteins was hindered by the absence of highly specific and sensitive assays able to measure levels in the circulation. Development of specific two-site assays for the different molecular forms of these proteins has facilitated the measurement of inhibin-A, inhibin-B, activin-A, activin-AB and follistatin in the circulation. The major source of dimeric inhibin-A and activin-A is the feto-placental unit. Therefore changes in serum

concentrations of inhibin-A and activin-A would reflect changes in placental synthesis and secretion. Recent studies in abnormal pregnancies suggest that these proteins may have a diagnostic role in the patho-physiology of human pregnancy. This chapter reviews the circulatory pattern, source, production and function of these proteins in normal and abnormal pregnancies.

5.2 Circulating Patterns During Pregnancy

5.2.1 Inhibin

Serum concentrations of immunoreactive (ir) inhibin has been reported to be higher during various stages of pregnancy compared with non-pregnant subjects (McLachlan *et al.* 1987, Abe *et al.* 1990, Tabie *et al.* 1991, Yohkaichiya *et al.* 1991, Baird & Smith 1993, Tovanabutra *et al.* 1993). In all of these previous studies ir-inhibin was measured using radio immunoassays (RIAs) which cross-react extensively with different inhibin α-subunit forms. Consequently, the contribution of the biologically active dimeric inhibin forms was unknown. In human pregnancy, inhibin-A is the major circulating form of inhibin with levels of inhibin-B near the detection limit of the assay (Muttukrishna *et al.* 1995, Fowler *et al.* 1998). As shown in Fig. 5.1, serum inhibin-A levels steadily decrease from eight weeks up to 16 weeks gestation. Levels remained low throughout the second trimester and then increased about five-fold during the third trimester and reached a maximum concentration at 36 weeks gestation. The major molecular form of inhibin-A in first, second and third trimester serum and term placental homogenate is 32 kDa (Muttkrishna *et al.* 1995).

Serial changes in the concentrations of inhibins A and B during the establishment of pregnancy have been reported by Illingworth *et al.* (1996). In spontaneous singleton pregnancies, inhibin-A levels start rising markedly from five weeks to reach a peak concentration at eight weeks gestation. After eight weeks inhibin-A levels gradually decline until 11 weeks gestation and remain low in the second trimester (Muttukrishna *et al.* 1995). Inhibin-B levels did not rise in early pregnancy and levels were near the detection limit of the assay (Illingworth *et al.* 1996).

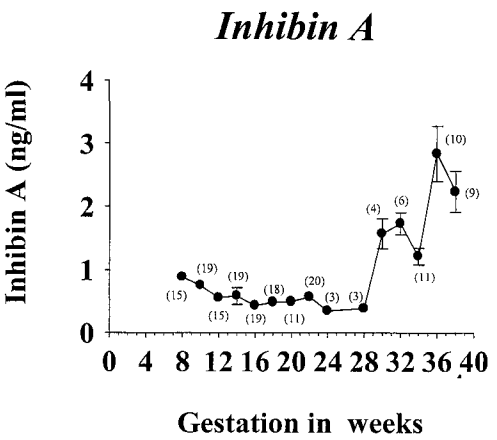

Fig. 5.1 Concentrations of inhibin-A (Mean ± SEM) in maternal serum during normal pregnancy (values in parantheses refer to numbers of samples assayed per time point). Data obtained from Muttukrishna *et al.* (1995), with permission.

5.2.2 Activin-A

Measurement of activin in circulation has been difficult due to the presence of the high-affinity activin-binding protein — follistatin. Despite the limitations, several groups have reported the measurement of activin in biological fluid using various assay formats including bioassay (Sakai *et al.* 1992); RIA (Robertson *et al.* 1992); enzyme immunoassay (EIA) (Woodruff *et al.* 1994, Wong *et al.* 1993) and competitive protein binding assay (Demura *et al.* 1993). However, the measurement of "total" activin-A (bound activin + free activin) in the circulation was only possible after the development of a sensitive, specific and accurate EIA (Knight *et al.* 1996). Using this assay, circulating concentrations of "total" activin-A have been measured throughout pregnancy. (Muttukrishna *et al.* 1996). Levels of activin-A did not vary significantly during the first and second trimesters. After 24 weeks, serum activin-A levels rise with a marked increase at term (Fig. 5.2).

The marked increase in inhibin-A and activin-A near term suggests a role for these proteins at parturition. Recent studies in the rat using a ligand

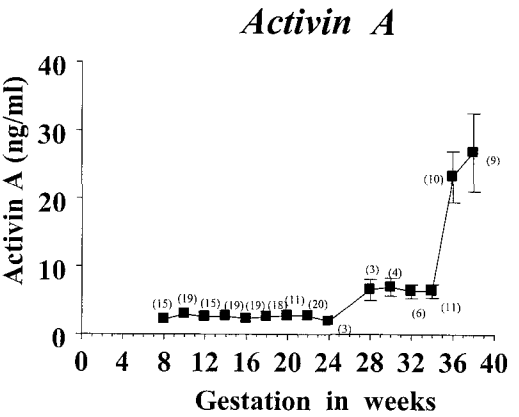

Fig. 5.2 Concentrations of activin-A (Mean ± SEM) in maternal serum during normal pregnancy (values in parantheses refer to numbers of samples assayed per time point). Data obtained from Muttukrishna *et al.* (1996), with permission.

binding assay have shown the presence of activin receptors in pregnant myometrium (Draper *et al.* 1997). Gel permeation chromatography indicated that all of the detectable activin-A in third trimester pregnancy serum and placental extract eluted with an apparent molecular weight between 200–700 kDa, indicating that little, if any, "free" activin-A is present in any of these samples.

5.2.3 Follistatin

Follistatin is a single-chain glycoprotein. There are different molecular forms of this protein as a result of post-translational modification. The major form of follistatin in the circulation contains 315 amino acids (FS315). In addition, follistatin containing 288 amino acids (FS288) is also present. *In vivo* and *in vitro* studies in the rat (Inouye *et al.* 1991) indicate that FS-288 is more potent than FS-315 certainly in suppressing follicle stimulating hormone (FSH) release. So although FS-288 may not be the major form in concentration terms, it may have quiet a significant biological effect. Various groups have

developed assays to quantitate different forms of follistatin with different specificities for the two forms (see Chapter 4). Follistatin is a high-affinity activin-binding protein. Therefore the bioavailability of activin will be determined by the concentrations of follistatin. During pregnancy, follistatin levels are markedly higher than in non-pregnant women. Maternal serum concentrations of follistatin. (FS288 and FS315) rise progressively with gestation (Fowler *et al.* 1998, Wakatsuki *et al.* 1996, respectively) suggesting that this increase in concentration was due to increased bulk of the placenta. During the first, second and third trimester of pregnancy, follistatin (FS315) levels are several fold higher than "total" activin-A, suggesting that bioactive "free" activin is not available in the circulation during pregnancy, confirming the gel permeation chromatography data (Muttukrishna *et al.* 1996).

5.3 Different Source(s) in Pregnancy

Human trophoblasts contain the machinery for the synthesis of inhibin/activin subunits and follistatin. However, various reported studies have shown that term trophoblasts express mRNAs for inhibin/activin subunits and follistatin (Petraglia *et al.* 1991, 1994) and subunit proteins are localised in the cytotrophoblast and syncytiotrophoblast of term villi (Petraglia *et al.* 1987, 1994, Minami *et al.* 1992). Villous cytotrophoblast cells express the mRNA for inhibin α-subunit, while activin β_A and β_B subunit mRNAs are localised both in the cytotrophoblast and syncytiotrophoblast cells (Petraglia *et al.* 1991). The maternal decidua and foetal membranes (amnion and chorion) express specific mRNAs and immunoreactive inhibin/activin subunits and follistatin (Petraglia *et al.* 1990, 1992).

A recent study using a monoclonal antibody against inhibin α-subunit has reported intense staining with inhibin α-subunit antibody in the syncytiotrophoblast in early pregnancy than in contrast to the much less intense staining seen in mature chorionic villi from third trimester gestations. There was weak staining in the cytotrophoblast and decidua (McCluggage *et al.* 1998). Minami *et al.* (1992) reported a positive immunostaining for inhibin subunits in the syncytiotrophoblast but not in the cytotrophoblast. In

the first trimester placenta, positive immunostaining for α_A and β_A subunits was clearly observed in the syncytial layer of the villi, whereas staining for β_B-subunit was faint. In the second trimester placenta, the relative intensities of staining for α_A and β_A subunits were similar to those in the first trimester with an enhanced positive immunostaining with β_B-subunit. The relative amount of inhibin α-subunit protein declined within the syncytiotrophoblast of the third trimester placenta whereas, β_A-subunit protein levels were unchanged (Minami *et al.* 1992).

Apart from the placenta, the granulosa and theca luteal cells in the corpus luteum of pregnancy exhibited positive staining with antisera directed towards α, β_A and β_B subunits, suggesting that the corpus luteum of the pregnancy might be involved in serum inhibin elevation early in gestation (Yamoto *et al.* 1991). Collectively, immunostaining reports published by various groups are not consistent and therefore not conclusive. In addition, the presence of subunit proteins or the mRNA does not confirm the production of biologically active dimeric proteins. Precise quantification of the dimeric proteins in the circulation and biological samples could be possible using the recently developed two-site ELISA (see Chapter 2).

Several *in vivo* studies have been carried out recently to confirm the source(s) of inhibin-A and activin-A in early pregnancy. After first trimester pregnancy termination (6–12 weeks), inhibin-A levels fall rapidly within an hour and the levels are similar to luteal phase levels within four hours of termination, suggesting the feto-placental unit as the major source of inhibin-A in early pregnancy. In the same study, activin-A levels also decrease more than 50% within four hours of termination suggesting a feto-placental source for activin-A. This study also confirms other source(s) for activin-A during pregnancy and/or a slow clearance rate in serum (Fig. 5.3; Muttukrishna *et al.* 1997a).

In donor egg pregnancies, in which there are no functional ovaries, inhibin-A and activin-A levels were measured in early pregnancy (5–12 weeks). When levels were compared to spontaneous singleton pregnancies, levels of inhibin-A were similar in both pregnancies confirming the feto-placental unit as the major source of inhibin-A in early pregnancy. However, activin-A levels were detectable in donor egg patients during the time of

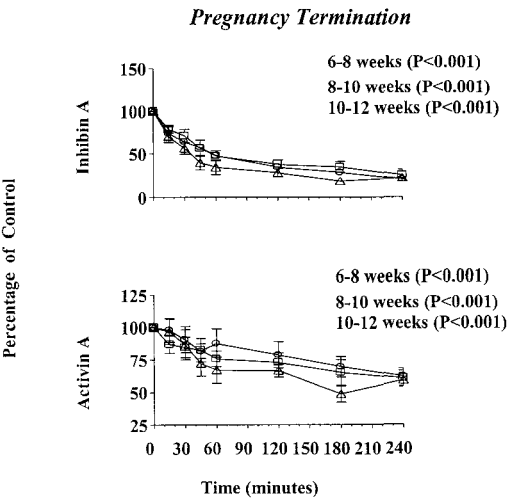

Fig. 5.3 Inhibin-A and activin-A concentrations (mean ± SEM) in peripheral plasma before and after the termination of pregnancy. Data are expressed as a percentage of the level before termination at 6–8 weeks - —□—, 8–10 weeks —○—, 10–12 weeks- —△—. $P < 0.001$ — One way analysis of variance. Data obtained from Muttukrishna *et al.* (1997a), with permission.

ovarian down-regulation and oestrogen and progesterone supplementation for endometrial growth suggesting extra-ovarian source(s) of activin-A including the endometrium (Birdsall *et al.* 1997). A recent study using immunostaining techniques has identified the presence of inhibin/activin subunits (α_A and β_A) in the human endometrium across the menstrual cycle. The α-subunit was localised in the luminal epithelium, glandular epithelium, stromal tissues and vascular endothelium with no significant variation across the menstrual cycle. The β_A-subunit common for inhibin-A, activin-A and activin-AB was localised in the luminal and glandular epithelium and in migratory cells while the endometrial stromal cells, decidua, vascular smooth muscle cells and endothelium were devoid of immuno-staining. The intensity of staining for β_A-subunit was faint in the proliferative phase and gradually increased during the secretory phase with the highest intensity in late secretory phase suggesting that activin-A may have a physiological role in endometrial function (Leung *et al.* 1998).

5.4 Placental Production of Inhibin, Activin and Follistatin

Placental homogenates contain high concentrations of dimeric inhibin-A (Muttukrishna *et al.* 1995) and activin-A (Muttukrishna *et al.* 1996). Using an assay specific for ir-inhibin (dimeric inhibins + monomeric α-subunit), two papers have reported the secretion of ir-inhibin (Qu *et al.* 1992, Qu & Thomas 1993) by placental trophoblasts. Term villous trophoblasts were cultured as reported by Kliman *et al.* (1986) and the secretion of ir-inhibin in the presence of various growth factors was studied. Immunoreactive inhibin secretion increased with time in culture up to four days. Epidermal growth factor (EGF) stimulated the secretion of ir-inhibin in culture. The addition of transforming growth factor α (TGF-α), transforming growth factor β (TGF-β), activin-A and human placental lactogen (hPL) individually at different doses did not affect ir-inhibin secretion. However, TGF-α and hPL had a synergistic stimulatory effect on ir-inhibin secretion. TGF-β and activin-A had a profound inhibitory effect on ir-inhibin secretion. Activin-A also suppressed hCG-induced ir-inhibin secretion by trophoblasts in culture. The inhibin assay used in the above studies does not distinguish between the different molecular forms of inhibin (either A or B), and is not specific for biologically active dimeric inhibins. The assay heavily cross-reacts with the monomeric inhibin α-subunit that is abundant in biological fluid and which has no known biological function to date.

Studies in our laboratory using the specific two-site ELISA for inhibins A and B in a similar cell culture system show that trophoblast cell culture medium contains detectable levels of dimeric inhibin-A while inhibin-B levels are undetectable, consistent with the serum measurements reported above. On the day of dispersion, almost 98% of the cells are cytotrophoblast and they differentiate into syncytiotrophoblast in culture within two days. Dimeric inhibin-A and activin-A levels are detectable from day 2 of culture and increase in concentration with time up to six days. This suggests that both cytotrophoblast and syncytiotrophoblast secrete inhibin-A and activin-A *in vitro*. EGF a growth factor that promotes cytotrophoblast differentiation has a dose-dependent stimulatory effect on hCG, inhibin-A and activin-A secretion. Inhibin-A and activin-A concentrations in the culture medium

positively correlate with hCG secretion, suggesting that these proteins are secreted mainly by the syncytiotrophoblasts (J. Asselin & S. Muttukrishna, unpublished observation). A recent study reported activin-A secretion by trophoblasts, amnion and decidual cells *in vitro*. Activin-A is secreted by all three gestational tissues and is stimulated in a dose-dependent manner by pro-inflammatory cytokines, tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) (Keelan *et al.* 1998).

Follistatin is also secreted by trophoblasts in culture. Secretion of follistatin increased in culture with time (studied up to 48 hours) (Petraglia *et al.* 1994). These studies provide direct evidence that dimeric inhibin-A, activin-A and follistatin are produced by placental trophoblasts during pregnancy. Further studies are required to study various factors that modulate the synthesis and secretion of these dimeric proteins from the placental trophoblasts.

5.5 Biological Function(s) in Pregnancy

It is now evident that high concentrations of biologically active dimeric inhibin-A and activin-A are in the maternal circulation during pregnancy. The biological importance of these proteins is as yet unclear. They may have an autocrine/paracrine effect on the placenta or on the developing embryo. There are no *in vivo* models available to study the role of inhibin and activin on placental hormone production. A major limitation is the high species specificity of placentation and the endocrinology of pregnancy, suggesting that experimental animals may not mimic human physiology. *In vitro* experiments provide evidence for autocrine/paracrine effect(s) of inhibin and related proteins in the placenta (Fig. 5.4). The addition of inhibin decreases while activin-A stimulates the release of hCG and progesterone from placental trophoblasts in culture (Petraglia *et al.* 1990, Mersol-Barg *et al.* 1990, Steele *et al.* 1993). Follistatin on its own has no effect. However, it reverses the effect of activin-A and gonadotrophin-releasing hormone (GnRH) on hCG and progesterone release in the placenta (Petraglia *et al.* 1994). Activin-A also increases the release of immunoreactive oxytocin from placental trophoblast (Petraglia *et al.* 1996).

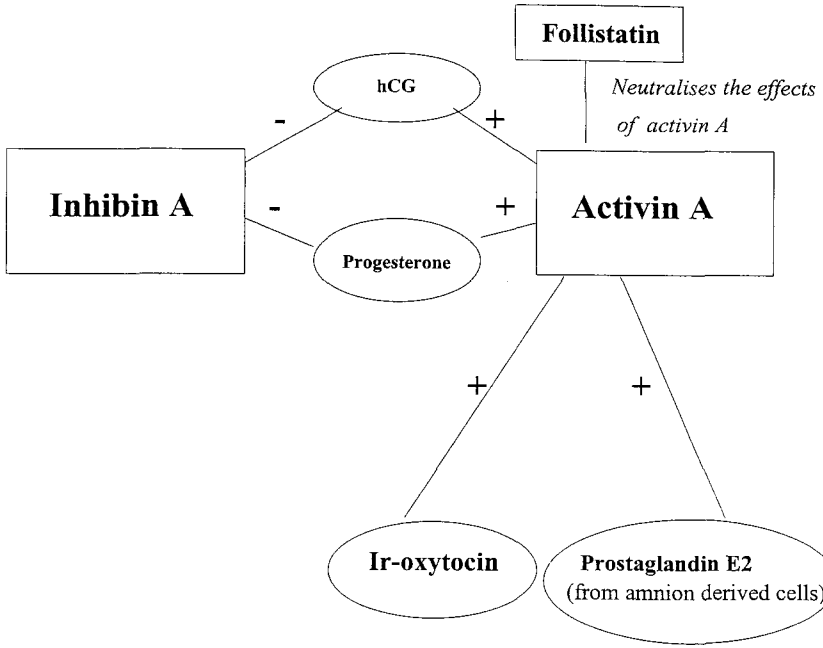

Fig. 5.4 Autocrine/paracrine effects of inhibin-A, activin-A and follistatin on the secretion of placental hormones *in vitro*.

In human amnion-derived cells, activin-A stimulated the release of prostaglandin E2 whereas inhibin did not have any effect on amniotic cells (Petraglia *et al.* 1993). When first trimester chorionic villi explants were cultured *in vitro*, addition of activin-A, not inhibin-A, stimulated the outgrowth of cytotrophoblast into the surrounding matrix. The outgrowth was characteristic of extra-villous cytotrophoblast cells *in vivo*. It was accompanied by cell division within the proximal region of the cytotrophoblast outgrowth, synthesis of fibronectin, as well as the expression of markers characteristic of invasive cytotrophoblasts cells. Activin-A also induced the early expression of Matrix Metallo Proteinase 2 (MMP-2) within villous cytotrophoblast. Addition of follistatin reversed these effects of activin-A, suggesting that activin-A promotes growth and differentiation in the villous cytotrophoblast (Caniggia *et al.* 1997).

Apart from the placental effects of these proteins, their effect on embryonic development has been studied in species other than the human. In mice, activin-A increases the rate of morula formation and the velocity of embryo cleavage (Orimo *et al.* 1996). Activin-A has been identified as the mesoderm inducing factor in amphibians and has a potent effect on embryo differentiation in Xenopus (Smith *et al.* 1990, Van Den Ejinden-Van Raaij *et al.* 1990). The expression of inhibin/activin β_B-subunit mRNA in the rat brain, in the areas of rapid cell proliferation surrounding the forebrain ventricles, suggest that the β_B gene may be involved in regulating neuronal cell growth and/or differentiation (Roberts *et al.* 1991). Collectively, these animal studies suggest that activin may have a role in embryo and foetal development during pregnancy. There are no reports on the effect of activin or inhibin on human embryo development. This is an area of research that warrants further investigation.

5.6 Abnormal Pregnancies

Recent studies using highly sensitive and specific assays for biologically active dimeric inhibin-A, inhibin-B, "total" activin-A and follistatin have shown that abnormal concentrations of inhibin-A and/or activin-A are in the maternal circulation in a variety of abnormal pregnancies suggesting that these proteins could be of diagnostic value in pregnancy (see Chapter 8).

5.6.1 Early Pregnancy Loss

The major source of inhibin-A in early pregnancy is the feto-placental unit. In IVF pregnancies, high levels of inhibin-A are detectable from four weeks [13 days following embryo transfer (ET)] with a peak concentration around ten weeks gestation. At four weeks gestation, significantly lower levels of inhibin-A were measured in patients who were subsequently diagnosed as missed abortions based on an ultrasound finding of a non-viable embryo, or an anembryonic gestational sac *in utero* four to six weeks after ET in the presence of continuing positive hCG pregnancy test (Lockwood *et al.* 1998).

This study suggests that a considerable amount of inhibin-A may be produced by the human embryo/foetus and/or the requirement of inhibin-A production by the placenta for the survival of the foetus in early pregnancy. This suggests that inhibin-A unlike hCG is not just a marker of the presence of trophoblastic tissue but could be a useful diagnostic tool in predicting viable ongoing pregnancies.

5.6.2 Down's Syndrome

Screening for Down's syndrome in the second trimester of pregnancy has been carried out with α-feto protein (AFP), unconjugated oestriol (UE3), hCG-β and intact hCG. Three studies using the α-subunit directed assays (Van Lith *et al.* 1992, Spencer *et al.* 1993, Cuckle *et al.* 1994) and four studies using the dimeric inhibin-A assays have shown that measuring maternal serum inhibin-A in second trimester would contribute to the existing screening test (Cuckle *et al.* 1995, Canick *et al.* 1994, Aitken *et al.* 1996, Wallace *et al.* 1996). Down's screening results were 77% predictive if inhibin-A was added as a fourth marker to the existing triple test (AFP, UE3 and hCG-β) and age from 15 to 18 weeks gestation. An advantage of using inhibin-A is the very small change in average inhibin-A from 15 to 18 weeks gestation in normal pregnancy. The inclusion of inhibin-A has significantly improved (by ~ 5%) the predictive value of the existing screening test. Amniotic fluid levels of inhibin-A levels are significantly lower in Down's syndrome pregnancies compared to chromosomally normal pregnancies from 16 to 19 weeks (Wallace *et al.* 1997). This suggests that the source of inhibin-A in the maternal serum may be different from that of amniotic fluid possibly with differential control of these sources.

Activin-A levels were not significantly altered in Down's syndrome affected pregnancies (Lambert-Messerlian *et al.* 1998). Inhibin/activin subunit gene expression studies have shown that inhibin α-subunit mRNA expression was significantly elevated in placental tissue (18 to 20 weeks gestation) of Down's syndrome affected pregnancies, suggesting that placental production of inhibin-A but not activin is increased in pregnancies affected by Down's syndrome.

5.6.3 Gestational Diabetes

Patients with gestational diabetes have an elevated concentration of serum activin-A which decreases with insulin therapy (Petraglia *et al.* 1995). Activin-A pulse frequency or amplitude is also significantly higher in patients with gestational diabetes and decreases after insulin therapy (Gallinelli *et al.* 1997), suggesting a possible modulatory role of glucose metabolism on activin secretion. Activin-A stimulates glycogenolysis in rat hepatocytes suggesting a role for activin-A in glucose metabolism (Mine *et al.* 1989).

5.6.4 Gestational Hypertension and Pre-eclampsia

Gestational hypertension is defined as sustained diastolic pressure $\geq$ 90 mmHg from previously lower levels, and pre-eclampsia is defined as gestational hypertension with sustained proteinuria $\geq$ 0.3 mg/24h. Inhibin-A and activin-A levels are significantly raised in pre-eclampsia compared to gestational age-matched controls (Muttukrishna *et al.* 1997b). All patients reported in this study developed pre-eclampsia around 30 weeks gestation. In early pregnancy (15 to 18 weeks gestation), there is a significant rise ($\sim$ 60%) in inhibin-A concentrations in pregnant women who developed pre-eclampsia later in pregnancy compared to normal pregnant women (Fig. 5.5) (Muttukrishna *et al.* 1997c, Cuckle *et al.* 1998). A longitudinal study shows that inhibin-A and activin-A levels are higher from 15 to 18 weeks in pregnant women who develop pre-eclampsia (< 34 weeks) than the normal controls. Pre-eclampsia patients who develop the disease at 34 to 37 weeks, have higher levels of inhibin-A and activin-A than gestation-matched controls from 21 weeks gestation (Fig. 5.6). Activin-A levels are also higher than the controls from 27 to 30 weeks gestation in pregnancies with gestational hypertension. Although the reason for the early rise in inhibin-A and activin-A in patients who develop pre-eclampsia is unclear at present, we could suggest that inhibin-A and activin-A are markers of placental dysfunction and may play a role in the patho-physiology of pre-eclampsia.

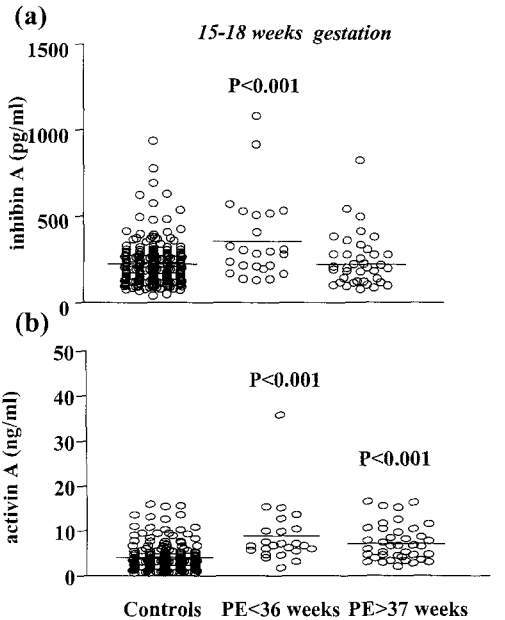

Fig. 5.5 Scatter plot of concentrations of **(a)** inhibin-A and **(b)** activin-A at 15 to 18 weeks gestation in normal pregnant women (control), women who developed pre-eclampsia pre-term (PE < 36 weeks) and women who developed pre-eclampsia at term (PE > 37 weeks).

5.6.5 Pre-term Labour

Maternal serum levels of activin-A are higher in pre-term labour patients and in pregnant women in labour at term than in term pregnant women not in labour (Petraglia *et al.* 1995). An increased activin-A release in the foetal compartment is also observed with an increase in β_A-subunit mRNA expression in pre-term labour (Petraglia *et al.* 1997). The addition of activin-A enhances the secretion of prostaglandin E2 from amniotic cells (Petraglia *et al.* 1993) and ir-oxytocin from placental cells (Florio *et al.* 1996). These findings support a role for activin-A in the mechanism of parturition.

Collectively, inhibin-A and activin-A levels in circulation are affected in abnormal pregnancies. The precise role of these proteins in human pregnancy

is still unknown. Therefore the reason for variation of these proteins in different gestational diseases are not explainable at present. Further detailed *in vitro* and *in vivo* studies are required to elucidate the factors controlling the production and, the biological role of inhibin-A and activin-A in human pregnancy.

5.7 Summary

- High concentrations of inhibin-A, activin-A and follistatin are present in the maternal circulation.
- Feto-placental unit is the major source of inhibin-A and activin-A in pregnancy.
- Placental cytotrophoblast and syncytiotrophoblast secrete inhibin-A and activin-A *in vitro*.
  - ➢ EGF stimulates the secretion of inhibin-A.
  - ➢ EGF, TNF-α and IL-1β stimulates activin-A secretion.
- Inhibin-A and activin-A have autocrine/paracrine effect(s) on placental hormonogenesis.
  - ➢ Activin-A stimulates placental secretion of hCG, progesterone, ir-oxytocin and prostaglandin E2. Follistatin neutralised these effects of activin-A.
  - ➢ Inhibin-A inhibits placental secretion of hCG and progesterone.
- Activin-A promotes growth and differentiation of cytotrophoblast in early pregnancy.
- In amphibians and mice, activin-A has autocrine/paracrine effects on embryonic development.
- Abnormal levels of inhibin-A and activin-A are in maternal circulation in some gestational diseases.

- ➢ Inhibin-A levels are raised in pregnancies affected with Down's syndrome and pre-eclampsia.
- ➢ Inhibin-A levels are lower at four to six weeks gestation in non-viable pregnancies.
- ➢ Activin-A levels are raised in pre-eclampsia, gestational diabetes and pre-term labour.

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CHAPTER 6

FOLLISTATIN: FROM PUBERTY TO MENOPAUSE

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6.1 Introduction

The purpose of this chapter is to review the current state of knowledge of follistatin in the context as a regulator of reproductive physiology. Therefore we will be concentrating on what might be perceived as the "classical" role of follistatin, whereas there is an ever-increasing body of literature documenting the actions of this protein in a variety of non-reproductive systems. For a broader coverage of these functions, the reader is referred to our recent review on this topic (Phillips & de Kretser 1998).

Follistatin was isolated as the third of a triumvirate of proposed endocrine regulators of follicle-stimulating hormone (FSH), the others being inhibin and activin. It was initially characterised in side fractions during the purification from follicular fluid of inhibin and activin, as another FSH inhibitory activity that had properties distinct from inhibin. Subsequently, two research groups published their isolation of this protein in 1987, one

naming it FSH-suppressing protein or FSP (Robertson *et al.* 1987) and the other group coining the term follistatin (Esch *et al.* 1987). With the subsequent availability of larger amounts of follistatin, it became clear that follistatin at pharmacological doses could inhibit FSH *in vivo* (DePaolo *et al.* 1991, Inouye *et al.* 1991, Meriggiola *et al.* 1994, Tilbrook *et al.* 1995). As discussed later, however, the role of follistatin as a significant endocrine regulator of FSH under normal physiological conditions is ill-defined.

6.2 Follistatin Structure and Mode of Action

Follistatin is encoded by a relatively small gene of around 6 kb and six exons, with an alternative splicing site that generates two major forms (see Fig. 6.1), giving a 344 amino acid pre-protein and a shorter 317 pre-protein where the sixth exon is spliced out (Shimasaki *et al.* 1988a, b). Removal of the signal peptide leaves mature peptides of 315 (FS315) and 288 (FS288)

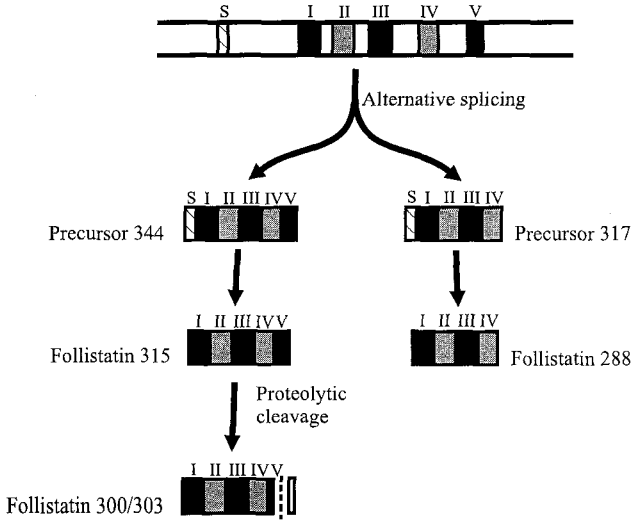

Fig. 6.1 Schematic of the follistatin gene and pathways of alternative splicing and proteolytic cleavage giving rise to the major forms.

amino acids, respectively. In addition, the 315 form of follistatin can be cleaved at the carboxy terminus by proteolytic enzymes to give a form of ~ 300 amino acids; this truncated form of FS315 is particularly abundant in follicular fluid (Esch *et al.* 1987, Inouye *et al.* 1991, Sugino *et al.* 1993).

A breakthrough in the understanding of follistatin's mode of action came with the observation that it is a high-affinity binding protein for activin (Nakamura *et al.* 1990). Estimates of k_D vary between 50–900 pM, which is in the general range of affinities reported for the binding of activin to its receptor (100–400 pM, see Phillips & de Kretser 1998). As such, follistatin can effectively compete with the activin receptor for activin ligand, explaining why follistatin is such a potent neutralising factor in a variety of cellular systems (Mather 1996).

Further biochemical characterisation of the follistatin forms indicated that the two splice variants, FS315 and FS288, had different properties. Although the various forms appear to have a similar binding affinity for activin (Sugino *et al.* 1993), it was noted that the FS288 variant had a high-affinity for heparin (Sugino *et al.* 1993, Sumitomo *et al.* 1995). Subsequent work from this group has led to the proposition that FS288 may be a mechanism whereby activin is targeted for degradation. Elegant experiments have shown that complexes of activin and FS288 become associated with cell surface proteoglycans through follistatin's heparin binding site, and thereafter are endocytosed and broken down by lysosomal enzymes (Hashimoto *et al.* 1997).

As alluded to above, FS315 has a similar affinity for activin, yet has a poor affinity for cell surface proteoglycans. Therefore, it has been proposed that FS315 is predominantly a circulating form, and there is some evidence to suggest it is the principal follistatin form in serum (Schneyer *et al.* 1996). This raises the question of what is the function of FS315 in the circulation? There are a number of possibilities, including a circulating store of follistatin, a means by which activin is targeted to specific tissues or cells, a mechanism by which activin escapes the degradative pathway via binding to FS288, or potentially another breakdown pathway that is yet to be delineated. This aspect of follistatin physiology is crucial to our understanding of these proteins and how they might regulate the bioavailability of activin.

6.3 Regulation of Follistatin

The regulation of follistatin is complex and appears to be co-ordinated in a tissue-specific manner. The promoter region of the follistatin gene contains DNA-binding elements consistent with regulation through both protein kinase A/cyclic AMP and protein kinase C pathways (Miyanaga & Shimasaki 1993). Consistent with its role as an activin-binding protein, follistatin mRNA is stimulated by activin (Bilezikjian *et al.* 1994), indicating a tight short-loop feedback system is in place to limit the biological activity of activin within a tissue. Further support for this regulatory loop comes from the observations that follistatin will inhibit the production of its own mRNA synthesis (DePaolo *et al.* 1993, Bilezikjian *et al.* 1996, Dalkin *et al.* 1996). A number of other factors are known to regulate follistatin mRNA in the pituitary-gonadal axis, and the reader is referred to the recent review for more comprehensive coverage (Phillips & de Kretser 1998). These include FSH and luteinizing hormone (LH) effects on follistatin synthesis from ovarian granulosa cells, epidermal growth factor in both granulosa cells and testicular Sertoli cells, and gonadotrophin-releasing hormone (GnRH) and steroids in cultured pituitary cells.

6.4 Follistatin Assays

A major complication in addressing the physiology of follistatin is the nature of the molecule itself, both in terms of its multiple forms and binding affinity for a number of dfferent substrates. This has hampered the development of antisera specific for the various follistatin species that measure reliably the bound or unbound fractions of follistatin. Nevertheless, assays have been developed with antibodies raised against recombinant follistatin preparations that have greater specificity for the FS288 splice variant (Saito *et al.* 1991, Evans *et al.* 1998, McConnell *et al.* 1998). Another approach has utilised follistatin's ability to bind to radiolabelled activin as an index of unbound follistatin present in a sample (Yokoyama *et al.* 1995), but this method does not take into account other activin-binding proteins such as α_2-macroglobulin (Mather 1996).

As well as the degree of specificity for follistatin forms, there is also the consideration of whether assays measure the total amount of follistatin (bound and unbound) or the unbound fraction only. With the former, either the antibodies which are used to detect follistatin are unaffected by activin (Khoury *et al.* 1995), or some sort of dissociating treatment is employed to remove the masking influences of activin (McFarlane *et al.* 1996, Phillips *et al.* 1996, Evans *et al.* 1998, O'Connor *et al.* 1999). The latter type of assay utilises antibodies that detect only follistatin unbound to activin (McConnell *et al.* 1998) or measures free follistatin by its ability to bind radiolabelled activin (Yokoyama *et al.* 1995).

6.5 The Conundrum of Follistatin in the Circulation

What is the nature of follistatin in the circulation and what purpose does it have? This question is easy to pose but far from easy to answer. As discussed earlier, there is evidence to suggest that FS315 is the predominant form in the circulation (Schneyer *et al.* 1996). Nevertheless, recent assays that are more or less specific for FS288 (Evans *et al.* 1998, McConnell *et al.* 1998, O'Connor *et al.* 1999) also detect measurable quantities of follistatin in serum. Furthermore, the lack of assays specific for the proteolytically cleaved ~ 300 amino acid form prevalent in follicular fluid (Esch *et al.* 1987, Inouye *et al.* 1991, Sugino *et al.* 1993) makes it unclear at present whether this form is present in significant amounts in the circulation.

Recent evidence from a number of groups shows that much of the follistatin detected in the circulation is in a bound state (Sakamoto *et al.* 1996, Woodruff *et al.* 1997, McConnell *et al.* 1998). The estimates vary from the studies depending on the particular assay utilised and the physiological state examined, but it appears that 70–90% of circulating follistatin is in a molecular complex. Although one is fairly safe in assuming that the majority of follistatin in these situations is bound to activin, there is evidence that follistatin can bind to other molecules such as α_2-macroglobulin (Phillips *et al.* 1997) and to other factors with structural homology with activin, such as inhibin (Shimonaka *et al.* 1991), bone morphogenetic protein (BMP)-4 and BMP-7 (Yamashita *et al.* 1995, Fainsod

et al. 1997). The relevance of these latter binding capabilities to reproductive physiology is yet to be explored.

As shown by a number of studies (see Phillips & de Kretser 1998), follistatin in the circulation is detectable and modulated by a variety of physiological states. Despite these clear demonstrations that circulating concentrations of follistatin are the result of some regulated process, what this represents is still somewhat of a mystery. Specifically, what is unclear is whether the follistatin in the circulation is an endocrine signal, as had been originally proposed when it was discovered, or does it reflect a means by which activin is maintained in a circulatory store. Other possibilities include whether the circulating follistatin is an index of what is occurring at the tissue level as part of a regulated process or may represent a circulatory complex that is destined to be metabolised in the liver or kidney and so has no active function. At the present time it is not clear which of these proposals are true, or whether all are to some extent correct. Nevertheless, there is emerging evidence that in many physiological states, follistatin is not an endocrine regulator of FSH, and this will be discussed below. The proposition that follistatin in the circulation represents specific tissue output(s) has some credence from the observations that some organs known to synthesise follistatin, such as the ovary and testis, contribute little to the circulatory concentrations (Klein *et al.* 1993, Phillips *et al.* 1996, Tilbrook *et al.* 1996), whereas other sources, such as the pituitary gland, may be significant secretory sites for follistatin (Phillips & de Kretser 1998).

6.6 Is Follistatin An Endocrine Regulator?

Since its discovery, follistatin was postulated to be an endocrine regulator of FSH, based on its ability to inhibit FSH production by cultured pituitary cells (Esch *et al.* 1987, Robertson *et al.* 1987). Furthermore, pharmacological administration of follistatin to animals confirmed the initial *in vitro* observations (DePaolo *et al.* 1991, Inouye *et al.* 1991, Meriggiola *et al.* 1994, Tilbrook *et al.* 1995), and indicate that if follistatin is sufficiently high in the circulation, it can efficiently inhibit FSH. Despite these seemingly clear-cut findings, there remains the paradox that follistatin under normal

physiological conditions does not appear to act in an endocrine fashion to regulate FSH. A number of lines of evidence suggest this: (i) the gonad is not a significant source of circulating follistatin (Klein *et al.* 1993, Phillips *et al.* 1996, Tilbrook *et al.* 1996), (ii) in women where gonadal function has been manipulated there is little change in serum follistatin concentrations (Gilfillan & Robertson 1994, Khoury *et al.* 1995), and (iii) experimentally-induced increases in circulating follistatin were able to inhibit FSH in gonadectomised rams (Phillips *et al.* 1996).

6.7 Follistatin as a Paracrine Regulatory Factor

Can the effects of follistatin on FSH and other reproductive processes be mediated through paracrine regulatory pathways? Perhaps the most elegant demonstration of follistatin as a paracrine factor comes from the recent study using mice over-expressing the follistatin gene (Guo *et al.* 1998). In all mouse lines over-expressing follistatin, there were clear decreases in testicular weight, whereas serum FSH levels were only inhibited in the F4 line, with the greatest over-expression of the gene (Fig. 6.2). Furthermore, there are clear indications in the follistatin transgenics that perturbations in the activin-follistatin system lead to spermatogenic arrest, seminiferous tubule breakdown and Leydig cell hyperplasia. These actions are likely to be mediated through paracrine regulatory pathways.

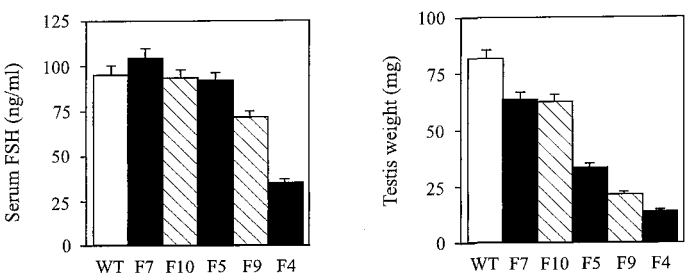

Fig. 6.2 Serum FSH and testis weights in wild-type (WT) and transgenic lines over-expressing the follistatin gene (F). Adapted from Guo *et al.* (1998), with permission.

In the ovary, follistatin also plays a crucial role in normal follicular development, as female mice over-expressing follistatin are infertile with under-developed uteri (Guo *et al.* 1998). In these animals, there are blocks in follicle growth at the pre-antral and antral stages dependent on the degree of follistatin over-expression, and in all animals there are no large antral follicles or corpora lutea. These findings are consistent with the expression of follistatin mRNA and protein, which is first observed in small antral and primary follicles respectively (McNatty *et al.* 1999). As with the testis, follistatin in the ovary is probably functioning as a paracrine mediator of the growth of early antral follicles.

Perhaps the best studied action of follistatin as a paracrine regulator is in the pituitary gland. Not long after its isolation, follistatin was found to be present in the pituitary gland (Gospodarowicz & Lau 1989, Kogawa *et al.* 1991a), leading to the supposition that follistatin might be acting as a intrapituitary regulator of FSH synthesis. With a number of subsequent studies, it is becoming clear that the paracrine regulation of FSH involves a complex interactive network between inhibin, activin and follistatin (Fig. 6.3), whereby

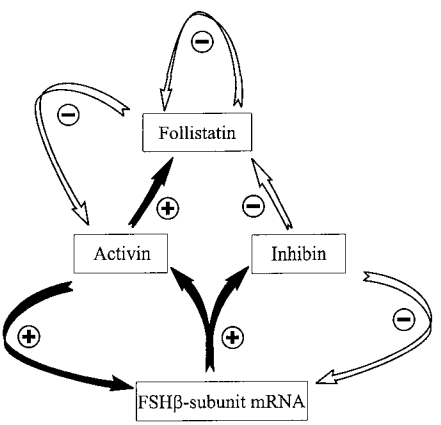

Fig. 6.3 Schematic representation of the paracrine inter-relationships that regulate FSH synthesis, with stimulatory streams indicated by black arrows and inhibitory streams by white arrows. Based on data from Carroll *et al.* (1989), DePaolo *et al.* (1993), Besecke *et al.* (1996), Bilezikjian *et al.* (1996) and Dalkin *et al.* (1996).

each component acts to balance the positive and negative signals controlling the synthesis of FSH β-subunit mRNA. For example, inhibin can signal directly to suppress FSH, but also inhibits the synthesis of follistatin mRNA, another negative regulator of FSH through its binding of activin. Conversely, activin directly stimulates FSH β-subunit mRNA, but at the same time feedback systems are activated by the activin's concurrent stimulation of follistatin mRNA, which will modulate this increased activity of activin to activate FSH. As indicated in Fig. 6.3, the documented action of follistatin to decrease FSH β-subunit mRNA (Carroll *et al.* 1989, Bilezikjian *et al.* 1996, Dalkin *et al.* 1996) is likely to occur through a neutralisation of the stimulatory effect of endogenous activin rather than a direct action of follistatin on the FSH gene.

6.8 Interlude

The preceding sections have dealt with the basic concepts of follistatin physiology and how these might relate to follistatin as an endocrine or paracrine regulatory protein. In the remaining part of this chapter, we will discuss studies relating follistatin to various aspects of human reproductive physiology. As discussed above, these studies are based on the assumption that a change in the circulating concentrations of follistatin represents an ordered process occurring in specific tissues.

6.9 Follistatin During Puberty

Until recently, determination of whether follistatin has an endocrine role during pubertal maturation has been hampered by the lack of specific and biologically relevant immunoassays. In determining if follistatin has an endocrine or paracrine role during puberty, one needs to take into consideration the nature of the assay utilised to measure follistatin, whether it detects total (activin bound + free follistatin) or free follistatin and the availability of activin, the signalling ligand which follistatin binds and inactivates.

The first report on changes in circulating follistatin during puberty came from the studies of Kettel *et al.* (1996). Utilising a homologous radioimmunoassay (RIA) (Sugawara *et al.* 1990) and an antiserum raised against porcine follistatin that detects the "free" follistatin as well as the activin-follistatin complex, these investigators reported that follistatin levels average approximately 5 ng/ml in circulation and remain unchanged during the course of sexual maturation in girls. A more recent cross-sectional study comparing the changes in follistatin levels during sexual maturation in girls showed that circulating patterns of follistatin varied with the assay systems utilised (Fig. 6.4); Foster *et al.* 2000). On the one hand, measurement of total follistatin levels using a heterologous RIA that utilises a rabbit polyclonal antiserum raised against 35 kDa bovine FS (O'Connor *et al.* 1999) and exhibiting 100% cross-reactivity to recombinant human follistatin 288 and 33% cross-reactivity for recombinant human follistatin 315, revealed that follistatin concentrations decrease progressively during pubertal maturation and are negatively correlated with advancing bone age. This is in stark contrast to the directionality of changes of another negative regulator of FSH, inhibin-A, which increased with progression of puberty (Foster *et al.* 2000). On the other hand, utilisation of a recently developed two-site ELISA assay that is specific for follistatin 288 but has 9% cross-reactivity for the 315 variant showed that follistatin concentrations peak during Stage III of puberty but decline thereafter. Interestingly, utilisation of a two-site assay

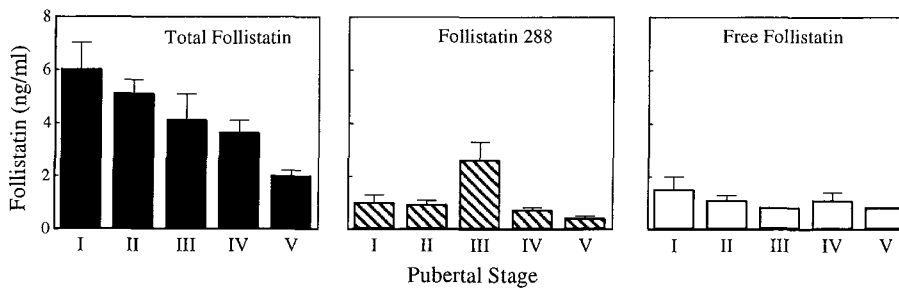

Fig. 6.4 Total follistatin, follistatin 288 and free follistatin (mean ± standard error) in girls across the pubertal transition (I–IV) and in follicular phase women (V).

that is specific for activin-free follistatin (the assay detects inhibin-bound follistatin) showed very little circulating follistatin (Fig. 6.4). Combined together these studies suggest that most of circulating follistatin is in an activin-bound state in the circulation and has raised questions regarding its endocrine role during pubertal transition.

Before one discounts an endocrine role for follistatin in puberty, however, it is necessary to consider the changes in follistatin in conjunction with changes in activin that occur during this time. Recent studies have shown that total concentrations of circulatings activin-A remain unchanged during pubertal progression (Foster *et al.* 2000). Information on changes in activins-B and AB are not yet available. The decline in follistatin, a binding neutraliser of activin, in the face of relatively constant total activin-A concentrations suggest that the bioavailable activin (free activin-A) may increase with pubertal progression such that activin could contribute to the increase in FSH that occurs during puberty. The final test for this premise requires knowledge of molar ratios of activin and follistatin in the circulation and the specific roles of the various follistatin variants. An issue that remains also to be addressed is what is the source of this circulating follistatin and whether the changes reflect the direction of paracrine changes within the pituitary.

6.10 Follistatin During Pregnancy

As in other physiological states, the importance of the changes in follistatin emerge from the demonstrated alterations that occur in the concentrations of activin, in particular activin-A. Several studies have established that activin-A is found in the placenta, the amniotic and allantoic fluids and the circulation of pregnant women (Meunier *et al.* 1988, Petraglia *et al.* 1991, de Kretser *et al.* 1994, Wongprasartsuk *et al.* 1994, Foulds *et al.* 1998). There is a progressive increase of serum activin-A levels in normal pregnancy which commences about 26 weeks and peaks at 38–39 weeks (Fig. 6.5; Muttukrishna *et al.* 1996, O'Connor *et al.* 1999). Furthermore, there is increasing interest in these findings as there is some evidence to suggest that activin-A concentrations may be abnormally high in women at risk of premature labour

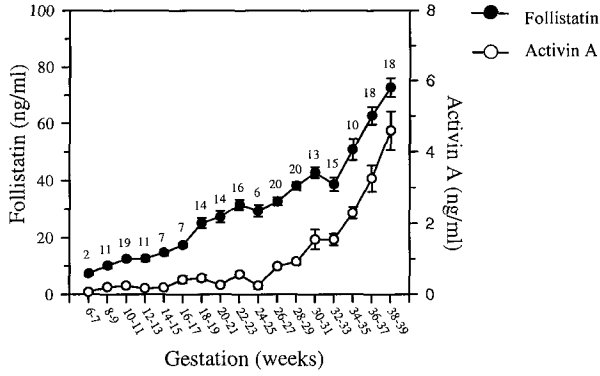

Fig. 6.5 Concentrations (mean ± standard error) of total activin-A (open circles) and total follistatin (solid circles) in the maternal circulation during human pregnancy (weeks 6–39). The number above each point indicates the number of samples per time point. Taken from O'Connor *et al.* (1999), with permission.

or in pregnancies complicated by *diabetes mellitus* (Petraglia *et al.* 1995, 1997).

Follistatin has also been isolated from human term placentae (de Kretser *et al.* 1994) and can be detected in ovine amniotic fluid (Wongprasartsuk *et al.* 1994). Several small studies have shown that serum follistatin levels rise during the 2nd and 3rd trimesters of normal pregnancy (Wakatsuki *et al.* 1996, Woodruff *et al.* 1997, Fowler *et al.* 1998). These data have been confirmed and extended in a larger cross-sectional and longitudinal study which showed that follistatin levels rose approximately six-fold from six to seven weeks to plateau at 30–33 weeks with a further significant sharp increase to term (O'Connor *et al.* 1999; see Fig. 6.5).

The significance of these changes in these two proteins remains unclear and requires further physiological data to clarify this issue. Since there is no sudden drop in follistatin levels, it is unlikely that a change in bioavailable activin levels occurs. It is possible that the role of the increasing follistatin concentrations is to block the biological actions of the increasing activin-A levels on the mother. Further studies will resolve these intriguing questions.

6.11 Follistatin During the Menstrual Cycle and During the Menopausal Transition

Unlike puberty where information regarding changes in follistatin is somewhat limiting and involve cross-sectional studies, changes in follistatin concentrations during the human menstrual cycle have received more intensive investigation and involved longitudinal studies. With the exception of one report which showed lower levels of follistatin during the luteal phase (Gilfillan *et al.* 1994), all other studies (Khoury *et al.* 1995, Kettel *et al.* 1996, McConnell *et al.* 1998, Evans *et al.* 1998), irrespective of the assay format utilised, report that circulating levels of follistatin remain constant throughout the cycle. The relative absence of activin-free follistatin during the human menstrual cycle as measured in the activin-free assay format (McConnell *et al.* 1998) suggest that most of the circulating follistatin exists in an activin-bound state. The unchanging nature of follistatin across menstrual cycle phases (Khoury *et al.* 1995, Kettel *et al.* 1996, McConnell *et al.* 1998), combined with the fact that the effects of follistatin appear to be mediated via neutralisation of activin activity, has led investigators to question an endocrine role for follistatin.

Changes in circulating follistatin levels during the menopausal transition have also been inconclusive and, as in the pubertal and menstrual cycle studies, have been highly dependent on the assay utilised. While Wakatsuki *et al.* (1996) reported age-related increases in circulating follistatin, other studies have reported no such increase (Khoury *et al.* 1995, Kettel *et al.* 1996). A recent cross-sectional study which utilised three different follistatin assays (Reame *et al.* 1998) found that follistatin levels are similar between young and pre-menopausal cycling women, although the absolute levels measured by the various assays differed considerably. While it is premature and perhaps erroneous to make quantitative comparisons across published reports involving different assay formats and standards, these data also bring to the forefront the complexity involved in estimating biologically meaningful levels of follistatin in the circulation.

Furthermore, while it is becoming increasingly evident that follistatin may not function as an endocrine regulator during the menopausal transition, the autocrine/paracrine relationships between activin and follistatin in

regulating the bioavailability of activin at target sites warrants further study. There is also very little information about a role for follistatin in a number of pathophysiological conditions. For instance, in women with polycystic ovarian syndrome, the intrafollicular follistatin concentrations were unchanged compared with normal women in one study (Erickson *et al.* 1995), whereas in a more recent study there is some evidence that the intrafollicular concentrations of follistatin may be perturbed in women with polycystic ovaries (Lambert-Messerlian *et al.* 1997).

6.12 Follistatin in the Male

The role of follistatin in the reproductive physiology of the human male is largely unexplored. A number of groups have published preliminary observations of serum follistatin concentrations in normal males (Gilfillan & Robertson 1994, Kettel *et al.* 1996, Sakamoto *et al.* 1996, Wakatsuki *et al.* 1996, Evans *et al.* 1998, McConnell *et al.* 1998), with the consensus being that there are no major differences in concentrations between normal males and females. There is a suggestion that follistatin concentrations increase with age in men (Wakatsuki *et al.* 1996) and that in pathological conditions such as primary testicular failure or hypogonadal hypogonadism, serum follistatin levels are unaffected (Gilfillan & Robertson 1994). However, these data sets are small and there is a definite need to revisit these areas in a more comprehensive manner. Interestingly, the levels of follistatin in seminal plasma appear to be around 30-fold higher than in the general male circulation (Anderson *et al.* 1998, Evans *et al.* 1998). Although the testis contains follistatin mRNA and protein as detected by *in situ* hybridisation and immunohistochemistry (Kogawa *et al.* 1991b, Kaipia *et al.* 1992, Majdic *et al.* 1997, Meinhardt *et al.* 1998), vasectomised men or men with oligo/azoospermia have equivalent concentrations of follistatin in seminal plasma compared with normal men (Anderson *et al.* 1998), demonstrating that the source of follistatin in seminal fluid is not the seminiferous epithelium. The human prostate gland contains significant amounts of follistatin protein as detected by immunohistochemistry (Thomas *et al.* 1997, 1998) and is likely to be the major contributor of follistatin in seminal plasma.

If the testis is not a significant contributor of follistatin either to the circulation (Phillips *et al.* 1996, Tilbrook *et al.* 1996) or seminal fluid (Anderson *et al.* 1998, Evans *et al.* 1998), does follistatin have a role within the testis? Although not well-defined, follistatin may act as an intratesticular paracrine agent to modulate spermatogenic and interstitial function (see Phillips & de Kretser 1998). Despite the relatively few studies addressing this question, the importance of follistatin in the testis can be judged from the observation that male mice over-expressing follistatin have gross morphological defects in their testes and are infertile (Guo *et al.* 1998).

6.13 Key Points and Future Prospects

In summarising this chapter, the following key points can be made:

- Follistatin has a number of forms generated by alternative splicing, differential glycosylation and proteolytic cleavage events, hampering the development of assays specific for the major forms.
- Most, if not all, of follistatin's actions are achieved through its high-affinity binding to activin, which neutralises activin's biological activity.
- The function of follistatin in the circulation is not well understood, but there is little evidence to suggest it is acting as an endocrine regulator of FSH.
- Follistatin's presence in a number of reproductive tissues suggests that it is an important paracrine/autocrine regulator of reproductive function.
- Concentrations of follistatin change during a number of reproductive stages, such as the menopausal transition, puberty and pregnancy, but the precise role of follistatin in these processes is yet to be determined.

Although some progress has been made in delineating the role of follistatin in human reproductive physiology, it is apparent that there is a host of

information yet to be gleaned. In part, our understanding of this protein has been hampered by assay development, such that assays specific for bound or unbound follistatin, and for the various molecular forms are only now becoming available. With these tools, it should be possible to expand upon the knowledge base presented in this chapter. Furthermore, the development of experimental models, such as follistatin transgenic animals, provides a powerful approach to study the reproductive systems where follistatin is proposed to be influential. There are interesting parallels between follistatin and inhibin, another of the triumvirate of FSH regulators mentioned in the introduction. It is only now with the development of specific assays for the inhibin forms that a further layer of complexity and specificity is being revealed whereby inhibin regulates human reproductive physiology (eg. Groome *et al.* 1996, Illingworth *et al.* 1996, Burger *et al.* 1998). It is quite conceivable that a similar situation will develop with follistatin once the appropriate methodologies become available.

Note Added in Proof:

Urbanek and colleagues (1999) have recently published a linkage analysis for genes associated with polycystic ovarian syndrome (PCOS). Of 37 candidate genes tested, the evidence for linkage with PCOS was strongest for follistatin. As noted in Sec. 6.11, the significance of follistatin in PCOS remains to be elucidated.

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CHAPTER 7

INHIBIN AND ACTIVIN IN THE MALE

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Inhibin-B is the major circulating form of inhibin in the male. Inhibin subunits are expressed in the testis early in foetal development and inhibin is present in the circulation in the neonate and during childhood, presumably as part of a mechanism for suppression of spermatogenesis and premature pubertal development. In the adult, inhibin and activin subunits can be identified within both the Sertoli and Leydig cell populations and a paracrine role for these peptides in the regulation of gonadal function has been proposed. Levels of inhibin-B in serum are tightly inversely correlated with follicle stimulating hormone (FSH) in the adult, with clinically useful correlates in male infertility management, although it does not appear that inhibin-B measurement should currently be recommended as a routine investigation in oligospermia. Circulating levels of inhibin-B fall with advancing age, as FSH rises. Inhibin and activin are also expressed within the prostate and may be involved in the pathogenesis of prostatic carcinoma and benign prostatic hypertrophy.

7.1 Introduction

Mottram and Cramer (1923) first hypothesised the very existence of "inhibin" on the basis of evidence collected from experiments conducted on male rats.

They observed that testicular irradiation resulted in hypertrophy of cells within the pituitary. Later, McCullagh in 1932 coined the term "inhibin" to describe the action of aqueous (and hence non-steroidal) testicular extract in eliminating these so-called "castration cells" in the rat pituitary. It took more than 60 years for science to move from these initial observations to the characterisation of the protein structure of the inhibin.

Much of this book describes evidence obtained using the various assays for inhibin in serum and biological fluids. The evolution of the sensitivity and specificity of these assays is well demonstrated by the recent history of our understanding of the actions of inhibin in the male. Thus earlier studies using the "Monash" iodinated radioimmunoassay allowed researchers to establish that immunoassayable inhibin did indeed exist in the male, as hypothesised by McCullagh many years earlier, and that levels rose at puberty, decreased gradually with ageing and fell below the limits of detection of the assay after castration (McLachlan 1987, McLachlan *et al.* 1988a, McLachlan *et al.* 1988b). Further work using this system suggested that circulating levels of inhibin in men were stimulated by FSH (and possibly also by the luteinizing hormone), but, disappointingly, that levels of immunoassayable inhibin did not reflect the varying degrees of testicular dysfunction as evidenced by measurements of conventional parameters of sperm number, function or quality. Earlier optimism that measurements of inhibin in serum might prove a sensitive and reliable means of assessing sperm dysfunction and possibly response to treatment proved ill-founded.

The development of new, highly sensitive and specific two site ELISA assays by Nigel Groome and his colleagues gave new impetus to inhibin research, particularly since assays specific for human inhibin appeared before those for animal species. For once, human research led the way in understanding of inhibin physiology, at least as reflected by fluctuations in serum levels of inhibin as markers for tissue function and endocrine/paracrine regulatory mechanisms. The ability of Groome's assays to distinguish between inhibins A and B rapidly resulted in the observation that inhibin-A was normally undetectable in the adult male (Illingworth *et al.* 1996), unlike the female in whom both species could be measured. Inhibin-B was the predominant species of dimeric inhibin in the male, although larger molecular

forms such as pro-α_C-containing inhibins were also present in significant amounts. The application of the inhibin-B assay in male human research further demonstrated a close relationship between levels of inhibin-B and FSH in the circulation in both healthy subjects and men with gonadal dysfunction (Illingworth *et al.* 1996) and suggested that levels of inhibin-B were indeed suppressed in oligospermic males, revisiting and validating the original "inhibin" hypothesis. Thus within ten years, the development of new methods of measurement had resulted in greater progress in understanding the role of "inhibins" in gonadal-pituitary inter-relationships than in the preceding 40.

This chapter will seek to define the current understanding of inhibin in the male, both in health at different stages of life, and in disease states such as male infertility and gonadal cancer. Progress is rapid, and the reader is directed to the website maintained by Professor Groome for regular updates of references in this area.

7.2 Inhibin in the Healthy Male

Inhibin α-subunit immunostaining appears in the rat testis as early as day 14.5 post-coitum, the day at which the testis can first be identified as a discrete structure (Majdic *et al.* 1997). Inhibin/activin β_B-subunit immunostaining appears slightly later but is similarly distributed within the foetal testis, suggesting that there is early assembly of dimeric inhibin-B pre-natally. Interestingly, foetal testicular expression of both α and β is mainly localised to Leydig cells although the major site of expression switches to the Sertoli cell in the neonate. In contrast, both inhibin/activin β_A and β_B subunits are detectable in the ovine foetus, in addition to α-subunit, but these are restricted to the Sertoli cell throughout gestation, appearing in the Leydig cell only between 40 and 90 days gestation (Jarred *et al.* 1999). In the human (Majdic *et al.* 1997), the anatomical distribution of immunostaining for the subunits differs, with localisation to the Sertoli and interstitial cells from the earliest detectable time of expression at 16 weeks gestation, although little work has been done on human tissue from the first trimester of pregnancy

using the new generation of sensitive monoclonal antibodies to the α and β_B subunits. The clearly detectable expression of α and β_B subunits at the time at which the testis is differentiating obviously suggests a role for inhibin-B as a putative regulator of the development of the organ.

Human infants are azoospermic or anovular and have small, apparently quiescent gonads. However, circulating gonadotrophins are elevated to high levels in the neonate (Winter *et al.* 1976, Forest *et al.* 1973) and perhaps unsurprisingly, levels of inhibin-B are also transiently raised in the neonate (Andersson *et al.* 1998). However, whilst FSH levels decline within nine months of birth, levels of inhibin-B remain elevated into the male adult range until much later in postnatal life, only falling some 12 to 18 months after birth. Maintenance of suppression of FSH and luteinising hormone (LH) is presumably necessary to avoid the premature onset of spermatogenic development and testosterone secretion, and it is possible that the maintenance of secretion of inhibin-B is one of a number of mechanisms by which the organism protects itself against these sequelae. LH appears to be suppressed earlier and more completely in the post-neonate, along with undetectably low levels of testosterone by six months of age. In contrast, FSH levels remain detectable and vary considerably between subjects, suggesting that maintenance of activation of the LH/testosterone axis would offer greater threat to the normal development and subsequent fertility of the infant.

7.3 Puberty and Adulthood

Spermatogenesis is initiated at approximately 13 years, at least as demonstrated by presence of spermaturia (Richardson & Short 1978). However, testicular enlargement, due largely to elongation of seminiferous tubules, begins much earlier. (Marshall & Tanner 1968). The initiation of puberty results in greatly increased secretion of testosterone and hence development of adult type secondary sexual characteristics. Longitudinal study of boys followed through puberty has demonstrated that dimeric inhibin-B levels increase sequentially from Tanner stage 1 to 3 (from early to mid-puberty) (Crofton *et al.* 1997), with strong positive correlation between

inhibin-B and FSH levels. This represents a dramatic switch from positive to negative feedback at puberty, resulting in a rapid attainment of adult range levels of inhibin-B. However, a larger cross-sectional study (Andersson *et al.* 1998a) has reported differing results, with attainment of adult levels of inhibin-B by Tanner stage 2, making inhibin-B secretion one of the earliest peri pubertal endocrine changes to reach adult levels in the male. The early rise in inhibin-B correlated with levels of LH and testosterone but not with FSH. This led the authors to suggest that early initiation of Leydig cell function in response to GnRH pulse driven LH secretion might be an important factor in the pubertal maturation and stimulation of the Sertoli cell. Further evidence for LH being a significant drive to testicular maturation and spermatogenesis comes from studies on men homozygous for the 566 C→T mutation, which inactivates the FSH receptor by an alanine to valine substitution in its extracellular domain (Tapanainen *et al.* 1997). Homozygous men exhibit varying degrees of spermatogenic failure but are neither azoospermic nor completely infertile. The results suggest that FSH is not essential for initiation of spermatogenesis, although it is possible that the FSH receptor retains some function in these individuals, albeit at a very low level.

Andersson *et al.* (1998b) who studied pre-pubertal boys and men with Sertoli Cell Only (SCO) syndrome further explored the nature of the cellular processes seen in the testis at puberty. They found that men with SCO had absent or barely detectable levels of inhibin-B, in contrast to pre-pubertal boys, in whom levels were equivalent to Tanner stage-matched healthy controls. When the same cohort of boys was re-studied after puberty, inhibin-B was undetectable. Using immunohistochemical evidence of staining for β_B-subunit in Sertoli cells in pre-pubertal testis; in germ cells and Leydig cells but not Sertoli cells in the adult, they hypothesise that mature Sertoli cells do not express the β_B-subunit, and hence men without spermatogenesis do not exhibit dimeric inhibin-B. The mechanism by which the switching of β_B-subunit synthesis from the Sertoli cell to the spermatocytes (and possibly Leydig cells) occurs remains to be determined, but may represent an important regulatory process in the initiation of puberty. Given the long duration of the birth–puberty interval in humans compared to rodents or sheep, this late alteration in site of synthesis of inhibin subunits in Man might reflect events that occur pre-birth in other species.

A number of groups have used the two-site ELISA assays for dimeric inhibin-B to study normal males. One problem that remains unresolved in the field of inhibin research is the lack of an internationally recognised reference standard against which assays can be calibrated. The difficulties engendered by this deficiency are well illustrated by the variation in the adult "normal" ranges quoted by different laboratories. At present, direct comparison between laboratories can, at best, be approximate, particularly given the many minor variations in assay technique that have been used in different centres. However despite between centre variations in absolute values, there seems to be a consensus that inhibin-B and FSH are inter-related in the adult male. Thus Anderson *et al.* (1997) demonstrated the inverse relationship between FSH and inhibin-B in a group of healthy men by weekly injection of depot testosterone. Testosterone treatment suppresses levels of FSH, LH and inhibin-B. Inhibin-B levels recovered slowly after treatment was discontinued, returning to the pre-treatment level weeks later than FSH, but concurrently with the return of spermatogenesis, at which time the inverse relationship with FSH was restored. Evidence that inhibin-B reflects spermatogenesis has accumulated from several studies of oligospermic males (described below), but the temporary disruption of the system with subsequent return to normal in this group of healthy men is convincing evidence for the "endocrine" nature of the inhibin B–FSH feedback loop in the absence of gonadal pathology. Later in life, inhibin-B levels in healthy men fall slowly but progressively from about the age of about 30 onwards (Byrd *et al.* 1998), mirroring a gradual rise in FSH, and consistent with the decreased levels of sperm production and serum testosterone seen in elderly men. This loss of gonadal peptides and steroids despite raised levels of FSH identifies an intrinsic decline in testicular function at both Sertoli and Leydig cell level.

It seems likely that the distribution of sites of synthesis of activin and inhibin within the testis is more complex than the classical separation between the Sertoli cell, as FSH responsive and inhibin secreting and the Leydig cell as LH responsive and testosterone secreting. Although immunohistochemical evidence of presence of subunits capable of dimerising into activin or inhibin must be treated with caution, and differences between species emphasised, it appears that at least in the rat, the Leydig cell is capable of expressing

inhibin-A and activin-A and the Sertoli cell inhibins A and B and activins A, B and AB (Kaipia *et al.* 1992, Wada *et al.* 1996, Majdic *et al.* 1997). Messenger RNA and subunit expression have also been described elsewhere in the male reproductive tract, at sites including the prostate, seminal vesicles and epididymis (Kaipia *et al.* 1994, Majdic *et al.* 1997, Roberts *et al.* 1997, Thomas *et al.* 1997, Ying *et al.* 1997, Jarred *et al.* 1999). Inhibin and activin appear to act antagonistically, at least *in vitro*, both in the regulation of spermatogonial mitosis and testosterone secretion form the Leydig cell (Franchimont *et al.* 1981, Lin *et al.* 1989, Mather *et al.* 1990). However, simple experiments observing the effects of castration on levels of inhibin in serum (Anawalt *et al.* 1996) and effects of vasectomy on levels of inhibin-B in seminal plasma (Anderson *et al.* 1998) clearly identify the testis as the major source of circulating inhibin-B in the human male. In contrast, activins are ubiquitous, being synthesised by a variety of tissues (Ying *et al.* 1997) and are viewed as paracrine rather than endocrine factors (Welt & Crowley 1998).

7.4 Disruption of the Inhibin B–FSH Feedback Loop

Some of the strongest evidence for a tight endocrine inter-relationship between inhibin-B and FSH in the male comes from studies of subjects undergoing chemotherapy or radiotherapy for treatment of cancer. As in the early studies of gonadal irradiation in the rat by Mottram & Cramer (1923) and McCullagh (1923), such treatment ablates spermatogenesis and induces a large rise in serum FSH. For example, Wallace *et al.* (1997) studied twelve men with haematological malignancy before and at monthly intervals during chemotherapy, and showed a halving in inhibin B levels within one month, with a nadir of approximately 20% of the pre-treatment value being reached by four months of therapy. The fall in levels of inhibin-B was mirrored by a rise in levels of FSH, which approximately doubled within one month and peaked by four months of treatment. This study elegantly demonstrates the close inter-relationship between the two hormones and identifies inhibin-B as a potentially useful marker of the degree of testicular damage following radiotherapy or chemotherapy. A long-term study demonstrating a rise in

inhibin-B as spermatogenesis recovers after cessation of treatment is now needed, to show whether inhibin-B measurement might be used as a tool to separate those men with good chances of natural conception after chemotherapy from those who are likely to have suffered permanent damage to sperm production and would be better advised to consider donor sperm treatment. Interestingly, Wallace *et al.* (1997) also found that the iodinated "Monash" radioimmunoassay failed to demonstrate a fall in "ir-inhibin", consistent with this assay's cross-reaction with circulating monomeric α-subunit, levels of which did not fall during chemotherapy. This observation explains previous confusion concerning the effects of chemotherapy on "ir-inhibin" which did not appear to fall as predicted after chemotherapeutic testicular insult (Brennemann *et al.* 1992). The maintenance of secretion of monomeric α subunits despite profound gonadal damage as seen in these studies, again casts doubt on the role of these species as regulators of gonadal function.

7.5 Restoration of Serum FSH in Hypogonadal Males

Hypogonadal hypogonadism is one of the most intensively studied disorders of the, hypothalamo-pituitary-gonadal (H-P-O) axis, representing a clear-cut "experiment of nature" in which absence or severe disruption of gonadotrophin-releasing hormone (GnRH) pulsatility induces hypo-secretion of gonadotrophins and consequent lack of gonadal stimulation. Individuals with the congenital variant, Kallmann's syndrome, fail to enter puberty and are subsequently infertile due to anovulation or azoospermia. In most cases, the remainder of the H-P-O axis retains function, so restoration of GnRH pulses using a mini-pump to administer appropriate doses of synthetic GnRH at pre-determined intervals will induce gonadal maturation, sex steroid synthesis and secretion and eventually restore fertility (Buchter *et al.* 1998). However, not all male patients with hypogonadal hypogonadism respond to pulsatile GnRH therapy by producing sperm, and others see restoration of spermatogenesis but only into the "severe oligospermia" range. In a manner similar to the use of early follicular phase inhibin-B measurement as a

marker for ovarian reserve in women (Seifer *et al.* 1997, Danforth *et al.* 1998), pre-treatment inhibin-B measurement has been used successfully to predict the success or failure of pulsatile GnRH treatment in hypogonadal men (Nachtigall *et al.* 1996). Restoration of GnRH pulsatility using long-term replacement therapy resulted in levels of LH, FSH and testosterone in the adult male range. Pre-treatment inhibin-B levels varied, with 30% of men having no detectable inhibin-B (limit of detection 15 pg/ml) but others having levels within the adult range despite well-documented hypogonadal hypogonadism. There were clear differences in response to therapy between those with low (< 60 pg/ml) and higher (> 60 pg/ml) levels of inhibin-B at baseline. Seventy per cent of those in the "higher" group went on to father children, and all ten subjects attained sperm concentration of at least 4 million per ml. In contrast, of the 12 men with pre-treatment inhibin-B levels below 60 pg/ml, only three attained sperm concentration > 4 million per ml or became fathers. This study has demonstrated a clear clinical use for inhibin-B assay in this particular condition — long-term pulsatile GnRH is a difficult and tedious treatment for patients to undergo, and accurate prediction of response will assist patients to decide whether to embark on such a regime.

The restoration of gonadal function in hypogonadal hypogonadism has also been used to illustrate the importance of the inhibin B–FSH feedback loop in the regulation of testicular function. Seminara and colleagues (1996) treated 18 men with idiopathic hypogonadotrophic hypogonadism with pulsatile GnRH and followed the subsequent increases in serum inhibin-B levels from a mean baseline of 68 pg/ml to 118 pg/ml after eight weeks of therapy. Levels of FSH fell in an inverse relationship with the rise in inhibin-B. Interestingly, the authors observed that patients who had experienced prior treatment with gonadotrophins had higher basal levels of inhibin-B, suggesting that treatment at a relatively early age might increase basal inhibin-B secretion. This observation should lead to study of the effects of early and late exposure of hypogonadal males to gonadotrophins, since it might be that programming of testicular function is influenced by peripubertal FSH, possibly improving the chances of achieving normal spermatogenesis later.

7.6 Inhibin-B and Male Infertility

The role of inhibin measurement in the clinical management of male infertility has been explored since the development of the "Monash" radioimmunoassay. However, results with this method proved disappointing, with no obvious differences between fertile and infertile men, excluding those with IHH (Lambert-Messerlian *et al.* 1994, Sheckter *et al.* 1988). Illingworth *et al.* (1996) were the first to report use of the two-site ELISA in infertile males, observing a close negative relationship between FSH and inhibin-B in cases of varying severity. The authors suggest that the assay might prove a useful serum marker of testicular function. A subsequent study (Anawalt *et al.* 1996) demonstrated that treatment of normal men with steroids (levonorgestrel and testosterone) suppressed, and exogenous FSH stimulated inhibin-B production. Inhibin-B was undetectable after orchidectomy and levels were subnormal in men with idiopathic oligospermia. These early studies clearly identified a physiological relationship between inhibin-B and FSH, and were followed by a number of clinical case series exploring the applicability of inhibin-B measurement in medical practice. There can be little doubt that a reliable and reproducible serum test of "sperm quality" would be useful; for example, in the prediction of success of testicular biopsy at retrieving sperm in cases of azoospermia. The widely used measurement of FSH to reflect preservation of spermatogenesis in such cases has been shown to be poorly predictive of successful sperm recovery (Ezeh *et al.* 1998) and inhibin-B assay in such cases has been shown to be a more sensitive marker for degree of spermatogenic impairment (von Eckardstein *et al.* 1999). However, the latter study also demonstrates that inhibin-B measurement alone or in combination with FSH could not reliably separate those men with true SCO syndrome (from whom no sperm could be retrieved), from those patients with partial SCO syndrome in whom repeated biopsies yielded sperm suitable for intracytoplasmic sperm injection (ICSI). However, other studies (Bohring & Krause 1999) have found lower levels of inhibin-B in patients with SCO syndrome than described by von Eckardstein *et al.* (mean levels of 15.8 pg/ml compared with 27.8 pg/ml), suggesting that von Eckardstein's study might have falsely identified some patients with foci of spermatogenesis as true SCO syndrome (fine needle biopsies can fail to

retrieve sperm in such situations, if foci of spermatogenesis are missed by the operator).

Inhibin-B measurement has also been suggested as an adjunct to FSH measurement in population studies of male fertility (Jensen *et al.* 1997). In this study, the combination of inhibin-B < 80 pg/ml and FSH > 10 IU/L was 100% predictive of a sperm count lower than 20 million per ml. Given current interest in possible decline in sperm density in Europe (Carlsen *et al.* 1992), these endocrine markers could prove useful in monitoring changes in testicular function over time in large epidemiological studies. An inverse relationship has also been shown between serum inhibin-B and sperm concentration in clinical cases of oligospermia (Klingmuller & Haidl 1997), with extremely low levels being identified in azoospermic men. However, although some studies suggest that inhibin-B measurement is superior to FSH in assessing degrees of male subfertility, others have cast doubt over the ability of a single "spot" measurement of inhibin-B to reflect accurately the level of impairment of spermatogenesis. Thus Pierik *et al.* (1998), using testicular volume and sperm count as a measure of "spermatogenic failure" found a close correlation between the degree of testicular failure and inhibin-B level, and that receiver operating characteristic analysis identified a better accuracy for inhibin-B than FSH in distinguishing competent from impaired spermatogenesis, whereas Bohring & Krause (1999), whilst confirming the observation that serum inhibin-B levels are correlated with testicular size and sperm count, were unable to conclude that measurement of inhibin-B was superior to FSH in identifying the degree of spermatogenic failure. Hence, although both Bohring & Krause (1999) and Klingmuller & Haidl (1997) identify men with severe oligospermia or azoospermia due to testicular failure as having very low levels of inhibin-B, this finding in itself is of limited clinical usefulness. Microscopic assessment of conventional sperm parameters — count, morphology and motility — will identify those individuals with sperm dysfunction so severe that their only realistic chance of pregnancy lies with ICSI. A clinically useful tool would identify gradations of sperm dysfunction allowing a more accurate prediction of the likelihood of pregnancy. Current evidence suggests that inhibin-B measurement does not add significantly to the predictive value of microscopic assessment of sperm quality plus measurement of FSH in milder forms of male subfertility.

7.7 Inhibin and Activin in the Prostate

Messenger RNA for both α and β subunits of inhibin have been demonstrated in both normal and malignant human prostate (Ying *et al.* 1997, Mellor *et al.* 1998, Thomas *et al.* 1998). In non-malignant prostate, inhibin α-subunit expression appears to be localised to the basal epithelium, and it is possible that both inhibin and activin act as paracrine regulators of epithelial cell proliferation. In high grade prostate cancer, inhibin α-subunit expression is down-regulated (Mellor *et al.* 1998) and a mechanism by which prostate tumour cells might over-ride a growth inhibiting effect of activin by secretion of follistatin FS288 has been proposed (McPherson *et al.* 1999). Thus an antibody which blocked FS288 allowed prostate cancer cells in culture to respond to addition of activin to the culture — an effect which was not demonstrated in the absence of the blocking antibody (McPherson *et al.* 1999). Evidence for the involvement of inhibin α-subunit in the regulation of a variety of gonadal tumours in the knockout mouse model has been presented in Chapter 11 of this volume. Further studies are needed to clarify this complex inter-relationship, but it seems likely that the TGF-β family of proteins will have an important role in the regulation of cell growth and proliferation in both normal and cancerous cell lines.

7.8 Summary

- Inhibin-B is the dimeric molecular form of inhibin in Man.
- Inhibin-B levels progressively increase through puberty in boys from early to mid-puberty, and has a positive correlation with FSH levels.
- Inhibin-B levels in adult men have an inverse relationship with FSH levels in circulation.
- Inhibin-B in men has a negative feedback effect on FSH.
- Inhibin-B levels are low in men with disorders of spermatogenesis.

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CHAPTER 8

MEASUREMENT OF INHIBIN AND ACTIVIN: A DIAGNOSTIC TOOL IN FEMALE REPRODUCTIVE ENDOCRINOLOGY?

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8.1 Introduction

The identification, purification and cloning of the members of the inhibin-activin superfamily and the subsequent development of sensitive and highly specific two-site enzyme-linked immunoassays for these polypeptide hormones has provided tentative answers to many of the outstanding questions concerning the regulation of the hypothalamo-pituitary-gonadal axis. Inhibin and activin are so closely related in structure, and so highly conserved across species, that their evolutionary significance seems undeniable. The presence of inhibin in a totipotential tissue such as placenta, which plays such a fundamental role and has such complex functions in supporting the growth of the foetus throughout pregnancy, inevitably raises questions about this other, non-ovulatory, function of inhibin.

8.2 Inhibin and Human Ovulation

Regular, cyclical mono-ovulation in women is achieved by a complex interaction of hormonal signals which prevent the "default to atresia" which

is the fate of 99.98% of the approximately two million primordial follicles which exist in the ovaries of the newborn female infant (Gosden 1985). Pre-antral stages of follicular growth occur independently of gonadotrophic stimulation (Baker & Scrimgeour 1991). However, antrum formation requires stimulation by follicle stimulating hormone (FSH), acting via its receptor in the granulosa cell surface membrane. From puberty, cyclic increases in pituitary FSH secretion rescue a cohort of follicles from atresia according to the "threshold" concept (Brown 1987). Although multiple follicles are recruited to begin pre-ovulatory development, as the FSH concentration rises at the beginning of each cycle, usually only one survives to become dominant — the follicle whose granulosa cells are most responsive to FSH. Development of the dominant follicle is characterised by the secretion of increasingly large amounts of oestradiol and inhibin-A into the circulation.

There is evidence to suggest that the maintenance of dominance is affected by intra-ovarian paracrine signalling (Hillier 1981) with inhibins and activins acting as important paracrine messengers (Hillier 1991). Inhibin-B rises from early in the follicular phase to reach a peak coincident with the onset

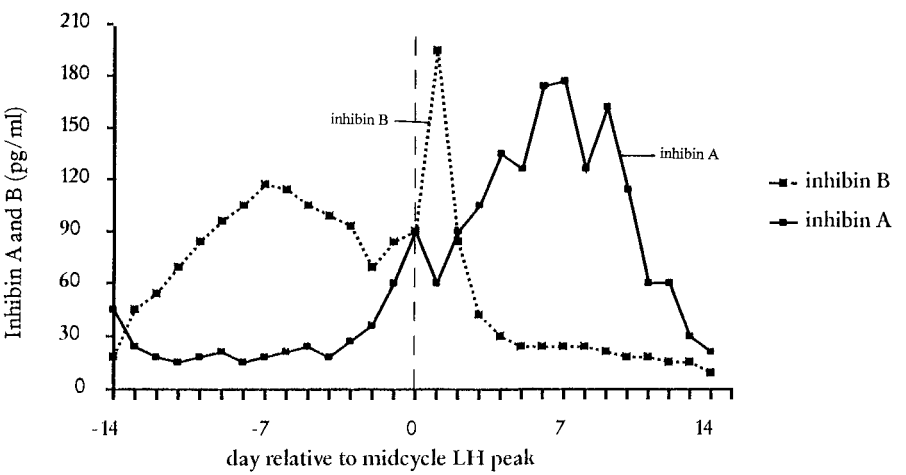


Fig. 8.1 Inhibins A and B during the menstrual cycle (data after Groome *et al.* 1996, reproduced with permission).

of the mid-follicular phase decline in FSH levels and then declines during the luteal phase apart from a peri-ovular peak which may represent release of follicular inhibin-B from the rupturing follicle into the circulation (Fig. 8.1). By contrast, inhibin-A levels are low in the early follicular phase, show a small mid-follicular phase peak, rise rapidly with ovulation and are maximal during the mid-luteal phase. During the luteal-follicular transition, inhibin-B concentrations rise rapidly to their mid-follicular peak whereas inhibin-A concentrations fall synchronously with oestradiol and progesterone to reach a nadir at the time of the intercycle FSH peak. The different patterns of circulating inhibins A and B during the two phases of the ovarian cycle are strong evidence for their playing different physiological roles during follicular recruitment, maturation and ovulation (Groome *et al.* 1996). Inhibin and activin thus provide a second, co-dominant system for modulating the ovarian cycle (in addition to oestradiol and progesterone) and they may be thought of as "fine-tuning" the hypothalamo-pituitary-ovarian axis.

8.3 The Inhibins and Activins in Dysovular States

Recent advances have been made in our understanding of inhibin physiology by studying naturally occurring dysovular states such as polycystic ovarian syndrome (PCOS), Kallmann's Syndrome and Resistant Ovary Syndrome. There have been conflicting reports in the literature concerning the inhibin status of women with PCOS, one of the most common endocrine disorders affecting ovulation (Clayton *et al.* 1992). The ultrasound definition of PCOS is the broadest, and using this definition PCOS may be identified in the majority of oligomenorrhoeic patients (87%) as well as in the majority of patients with hirsuitism including those with ovulatory cycles (87%), and in 32% of amenorrhoeic patients. Hyperinsulinaemia is present in many women with PCOS, even in the absence of obesity, suggesting that some metabolic disorder may contribute to the condition (Morales *et al.* 1996, Hopkinson *et al.* 1998). The aetiology of PCOS is unclear, but its effective treatment by both anti-oestrogens and by exogenous FSH suggests that a primary disorder of FSH regulation may be central.

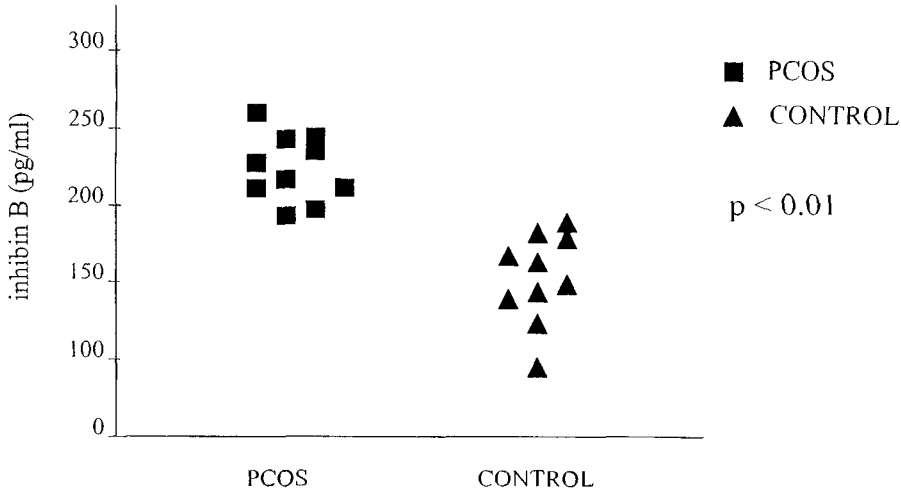


Fig. 8.2 Scatter plot of individual concentrations of serum inhibin-B levels on cycle day 5 in PCOS patients (n = 10) and ovular controls (Lockwood *et al.* 1998a).

To investigate a possible role of inhibin-B in the pathophysiology of PCOS, serum inhibin-B levels were measured in women with PCOS on cycle day 5 of a spontaneous or progestogen-provoked bleed and compared with levels on cycle day 5 of women with regular, ovulatory cycles. The mean serum inhibin-B levels in the PCOS patients were significantly higher (Lockwood *et al.* 1998a) compared with normal controls and this finding is consistent with the exquisite sensitivity to exogenous FSH seen in PCOS patients undergoing fertility treatment (Fig. 8.2).

Serial blood sampling on cycle day 5 of women with clomiphene-resistant PCOS and normal controls revealed that in ovular women, there is a distinct periodicity in inhibin-B levels with a clear peak detectable every 60–70 minutes (Lockwood *et al.* 1998a) whereas in women with ovulatory dysfunction due to PCOS, no such pattern of regular pulsatility was seen (Fig. 8.3).

It is recognised that the development of large antral follicles can be stimulated by the injection of very small amounts of FSH, suggesting that there is a relative deficiency of FSH in women with PCOS. This deficiency

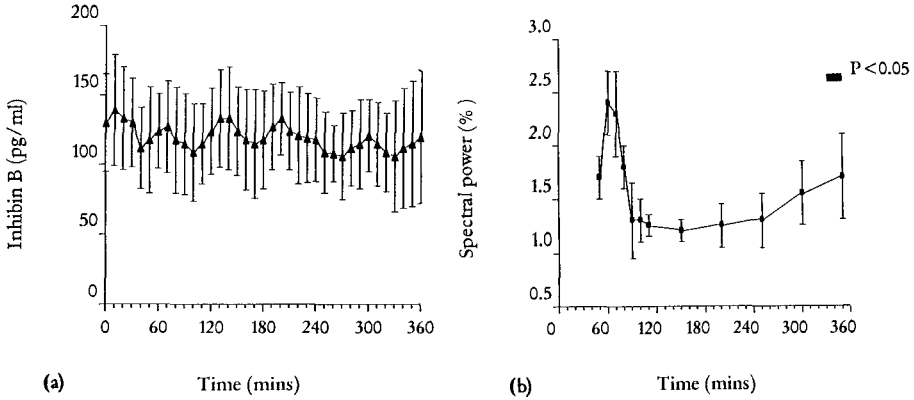


Fig. 8.3 **(a)** Mean serum inhibin-B levels in normal controls (n = 54). **(b)** Fourier Transform of the data (data from Lockwood *et al.* 1998a, with permission).

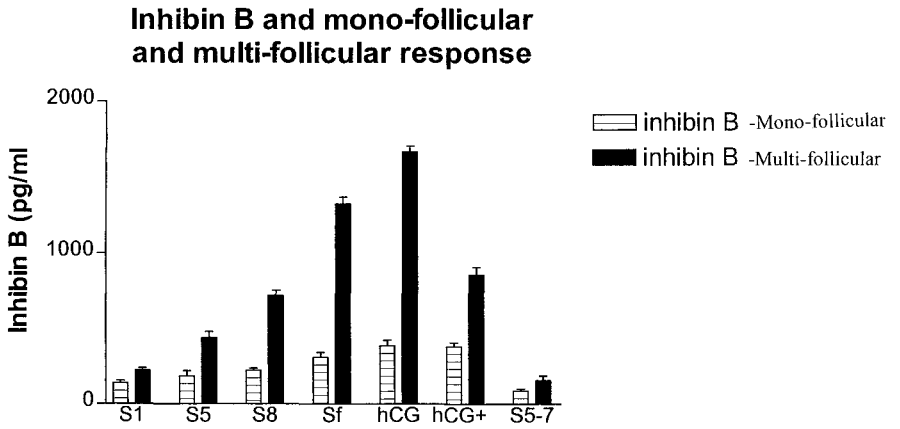


Fig. 8.4 Inhibin-B levels during ovulation induction with recombinant FSH in patients with PCOS (n = 15). S_1, S_5 and S_8 = 1st, 5th and 8th day of stimulation; Sf = day on which at least one follicle ≥ 12mm seen; hCG, hCG +1 = day of hCG administration and day after; S_{5-7} = luteal phase, 5–7 days after hCG.

may express its effects during the crucial intercycle rise in FSH which is seen in spontaneous ovular cycles and plays an essential role in promoting further development of the ovulatory follicle (Yen *et al.* 1970, Baird *et al.* 1977, Hamilton-Fairley *et al.* 1991).

If PCOS is interpreted as a disease of inappropriately low FSH secretion (Yen *et al.* 1970), then we can explain the characteristic hormone profile of low/normal FSH in conjunction with elevated LH as a response to elevated inhibin-B secretion. The "elevated inhibin-B" hypothesis therefore, whether it represents an initiation of or a response to pituitary-ovarian dysfunction, offers a unifying neuroendocrine explanation for the aetiology of PCOS.

The problems associated with ovulation induction with exogenous gonadotrophins in women with PCOS are due to their tendency to recruit

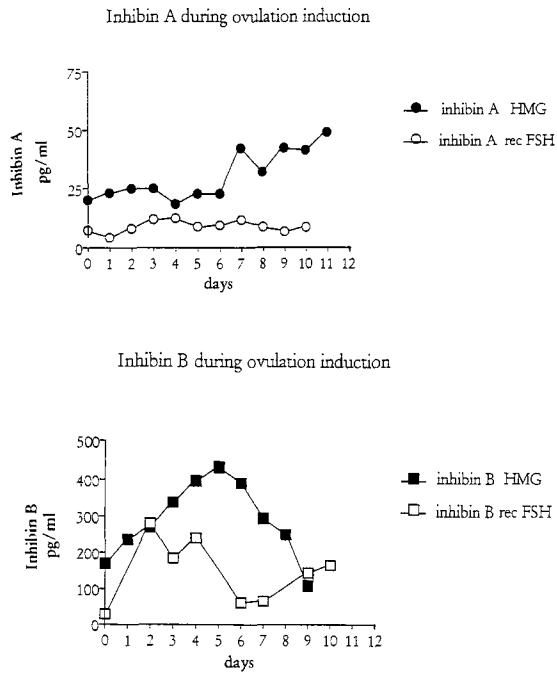


Fig. 8.5 Inhibins A and B levels during ovulation induction with recombinant (rec) FSH in Kallmann's Syndrome.

multiple follicles and conceive multiple pregnancies and/or develop OHSS. We have recently reported evidence suggesting that inhibin-B levels in PCOS patients who respond to ovulation induction with mono-follicular development are significantly lower than those of PCOS patients who recruit multiple follicles (Lockwood *et al.* 1999) (Fig. 8.4).

Hypogonadotrophic states such as Kallmann's Syndrome provide an opportunity to explore the relationship between circulating levels of inhibins, activins, gonadotrophins and steroids during stimulated folliculogenesis. We have previously demonstrated that ovarian stimulation of women with no endogenous FSH or luteinising hormone (LH) secretion due to Kallmann's Syndrome with recombinant FSH (with no LH activity) results in the development of ultrasonographically normal but functionally abnormal follicles with very low oestradiol and inhibin levels (Lockwood *et al.* 1996b) (Fig. 8.5). From these data, we can conclude that FSH is able to induce follicular development until the pre-ovulatory stage, but in the total absence of LH, the follicle does not produce oestradiol nor become a functional corpus luteum. The aberrant pattern of inhibin and activin secretion seen in the recombinant FSH cycle confirms the absolute requirement for some LH for functional folliculogenesis to occur and thus confirms the "two-cell, two-gonadotrophin" theory of folliculogenesis.

8.4 Inhibin and Activin in Superovulated Cycles

Gonadotrophin-releasing hormone (GnRH) analogue (pituitary desensitised) treatment cycles are widely used in assisted reproductive therapies such as Gamete intrafallopian transfer (GIFT) and *in vitro* fertilisation (IVF). The benefit from the use of GnRH analogues in IVF derives principally from a reduction in the incidence of premature LH surges and hence premature luteinisation (Dor *et al.* 1992, Hughes *et al.* 1992, Smitz *et al.* 1992), resulting in a lower probability of cycle cancellation and higher chances of embryo transfer and/or embryo cryopreservation per cycle started (Chetowski *et al.* 1989, Tummon *et al.* 1992). Endometrial receptivity may also be enhanced, resulting in an increased implantation rate in association with GnRH analogue

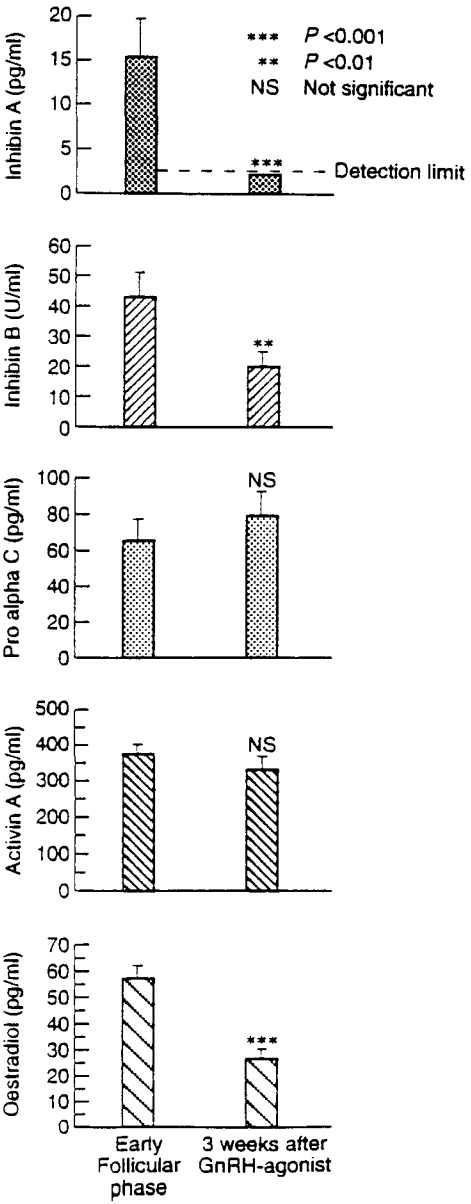


Fig. 8.6 Effect of GnRH agonist treatment on levels of inhibin-A, inhibin-B, pro-α_C, activin-A and oestradiol (data from Lockwood *et al.* 1996a with permission).

use (Testart *et al.* 1989). It has been suggested that increased fecundity with GnRH analogue pituitary desensitisation may also be associated with a widening of the "implantation window" following embryo transfer (Tur-Kaspa *et al.* 1990). Pituitary desensitisation produces a marked suppression of inhibin-A with a more modest suppression of inhibin-B and no significant effect on inhibin pro-α_C or activin-A when compared to normal early follicular phase levels (Lockwood *et al.* 1996a) (Fig. 8.6).

Stimulation with FSH produces large increases in inhibins A and B and pro-α_C with highly significant correlations being seen between the number

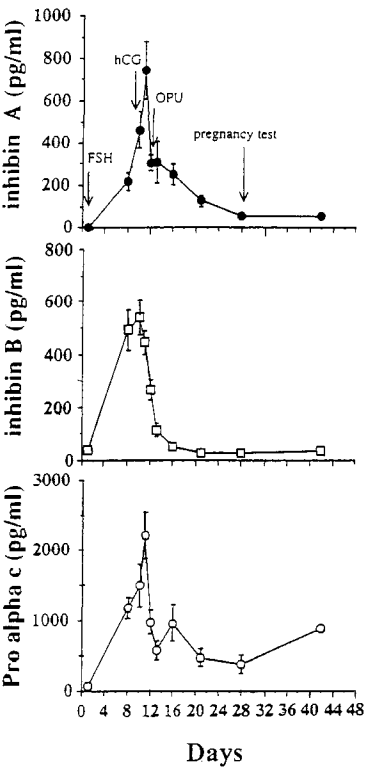


Fig. 8.7 Circulating concentrations of inhibin-A, inhibin-B and pro-α_C during ovarian stimulation with FSH. Values are mean ± standard error of the mean (SEM). OPU = day of oocyte pick-up, FSH = 1st day of FSH, hCG = day of ovulatory trigger (data from Lockwood *et al.* 1996a, with permission).

of large follicles developing and inhibin-A, pro-α_C and oestradiol levels (Fig. 8.7). There were significant increases in oestradiol concentrations, but levels of activin-A remained unaltered after 7–8 days of FSH and then rose significantly on the day of human chorionic gonadotrophin (hCG) administration. Peak levels of inhibin-A and pro-α_C were reached on the day after hCG treatment (while levels of inhibin-B were already falling at this time). The luteal phase was characterised by a further decline in inhibins A and B, pro-α_C and oestradiol. It was found that there was a significant relationship between the number of follicles developing, number of oocytes retrieved and the levels of inhibins and steroid hormones observed.

The rise in inhibin during gonadotrophin stimulation corresponds to earlier observations during cycles stimulated with clomiphene citrate-human menopausal gonadotrophin (HMG) (Mc Lachlan *et al.* 1987a), and without GnRH analogue pretreatment (Hughes *et al.* 1990) and adds weight to the suggestion that measurement of inhibins might indicate the level of follicular recruitment and maturation during controlled ovarian hyperstimulation cycles and possibly be a means of predicting and monitoring ovarian hyperstimulation syndrome (OHSS) (Welt *et al.* 1997). Studies of inhibin-B in hyperstimulated cycles have shown that very high levels (>1000 pg/ml) are generated in the presence of multiple follicles (Lockwood *et al.* 1996a) whereas the mid-follicular phase rise in inhibin-B in spontaneous cycles originates from the few pre-dominant follicles. In superovulatory cycles, the multiple co-dominant follicles are presumably responsible for the high levels of inhibin-B found and this observation is consistent with studies showing that small follicles express more β_BmRNA (Roberts *et al.* 1993).

8.5 Inhibins During the Perimenopause

The period of time leading into the menopause in women, the perimenopausal transition, is often characterised by cycle irregularity and is marked by dramatic changes in the hypothalamo-pituitary-ovarian axis. The earliest and most consistent reproductive endocrine finding associated with reproductive ageing in women is a selective rise in circulating FSH levels unaccompanied

by a rise in LH (Klein *et al.* 1996). The resultant ovarian changes include short follicular phases with early ovulation and luteal insufficiency characterised by lower levels of progesterone secreted for shorter periods of time compared to the luteal phase of younger women (Treloar *et al.* 1967). Klein found that older ovulatory women have a monotropic FSH rise throughout the menstrual cycle without any changes in the bioactivity of the FSH molecule or GnRH pulsatility. Such women show an earlier onset of the intercycle FSH rise, shorter follicular phase length associated with an earlier recruitment of a dominant follicle and an earlier rise in ovarian oestradiol production.

However, changes in ovarian steroid secretion adequate to account for the rise in FSH seen during early reproductive ageing have not been noted. Since FSH levels are co-regulated by inhibin, it has been suggested that decreased secretion of ovarian inhibin (Sherman & Korenman 1975) by the decreasing follicular pool (Richardson *et al.* 1987, Faddy *et al.* 1992) may be primarily responsible for the monotropic rise in FSH seen in older women. Studies using earlier, non-specific inhibin assays did not demonstrate a clear association between early follicular phase FSH and inhibin (Lenton *et al.* 1991), but using the ELISAs that distinguish between inhibin-A, inhibin-B and the monomeric subunit, Klein (Klein *et al.* 1996) & Danforth (Danforth *et al.* 1998) have shown that both luteal phase inhibin-A and follicular phase inhibin-B levels are inversely correlated with follicular phase FSH levels.

In a large prospective longitudinal study of the perimenopausal transition, Burger *et al.* found that the major significant endocrine event in women in the early perimenopausal phase of the transition is a substantial fall in the circulating levels of inhibin-B, with no significant change in inhibin-A or oestradiol levels (Burger *et al.* 1998). Schipper (Schipper *et al.* 1998) undertook a study to investigate whether variable endogenous FSH concentrations, as have been observed in normo-ovulatory women (van Santbrink *et al.* 1995), are related to menstrual cycle characteristics. It was found that there was a lack of correlation between maximum FSH levels and cycle characteristics, and it was not possible to confirm the proposed relationship between increased FSH concentrations and a relative shortening of the menstrual cycle as a consequence of a diminished ovarian reserve.

Santoro and co-workers (Santoro *et al.* 1999) have recently examined the hypothesis that the increased FSH levels noted in older women during the years preceeding the perimenopausal transition is due to both decreased inhibin and increased activin-A secretion. They demonstrated that follicular phase inhibin-B and luteal phase inhibin-B were reduced in older versus younger women, and activin-A was elevated throughout the cycle of older versus younger women. They concluded that lack of restraint by inhibin A and B contributes to the FSH rise associated with reproductive ageing and that activin-A may also play an endocrine role in maintaining elevated FSH in older reproductive aged women.

Demographic changes have increased the average age at which women pursuing fertility treatments are undergoing Assisted Reproductive Techniques (ARTs), and older women often obtain poor oocyte numbers which are associated with low pregnancy rates. Poor response is not restricted to older women, however, and given the emotional, psychological and financial costs associated with ART, it is important that women considering fertility treatment are appropriately counselled about their individual chances of a successful outcome.

Evaluation of ovarian reserve prior to initiation of ovarian stimulation by measuring basal or stimulated FSH concentrations in the early follicular phase has been thought to provide prognostic information about chances of success with fertility treatment (Fanchin *et al.* 1994, Hansen *et al.* 1996, Kim *et al.* 1997, Sharif *et al.* 1998). Sharif and colleagues demonstrated that for IVF treatment following pituitary desensitisation, basal FSH was a better predictor of cancellation rate and of the number of oocytes collected than age, but age was a stronger predictor of pregnancy rate. Many other prognostic factors have been shown to correlate with the chances of achieving pregnancy with IVF treatment including duration of infertility, parity, age and previous treatment cycle outcome (Templeton *et al.* 1996). However, even after allowing for age, parity, follicular phase FSH levels, Body Mass Index (BMI), previous pelvic surgery and the presence of significant endometriosis, ART programmes frequently encounter patients whose treatment cycles are surprisingly sub-optimal or need to be cancelled because they have under responded to gonadotrophin stimulation.

Given the clear deficiencies of standard tests in predicting ovarian response to FSH superovulation, several groups have assessed the performance of early follicular phase inhibin-B measurement in identifying women with a reduced ovarian pool of primordial follicles.

8.6 Inhibin-B and the Assessment of Ovarian Reserve

The observation that women with PCOS (who are notoriously recognised to be at risk of over-responding to gonadotrophin stimulation during ART) have significantly raised levels of inhibin-B (Lockwood *et al.* 1998a, Anderson *et al.* 1998) provided support for the hypothesis that inhibin-B levels, as a product of ovarian granulosa, might be an indicator of the number of pre- and small antral follicles early in the follicular phase. Success with *in vitro* fertilisation-embryo transfer (IVF-ET) treatment is related to the ability to produce large numbers of ovarian follicles in response to stimulation with exogenous FSH, and hence IVF success rates may be considered to be a function of ovarian reserve.

In the study by Seifer *et al.* (1997), a low day 3 serum inhibin-B concentration was predictive of poor response to ovulation induction and of decreased success during IVF treatment cycles. Inhibin-B concentrations were found to increase within follicular fluid as growth during the ovarian cycles proceeded.

Lockwood *et al.* (1997a) also found that mid-follicular phase (cycle day 5–6) levels of inhibin-B were prognostic of unexpected over and under response to gonadotrophin stimulation in IVF treatment cycles. In this study, 40 patients under 40 years of age with normal FSH levels undergoing a first cycle of IVF had mid-follicular phase serum samples obtained prior to commencing pituitary desensitisation with GnRH analogue. Twenty-seven (60%) patients were regarded as normal responders to gonadotrophin stimulation, but five patients were cancelled due to poor response and eight had an excessive response requiring cycle cancellation or all the resultant embryos were frozen due to risk of OHSS. The mean serum inhibin-B levels for the poor and excessive responders (<85 pg/ml and >250 pg/ml

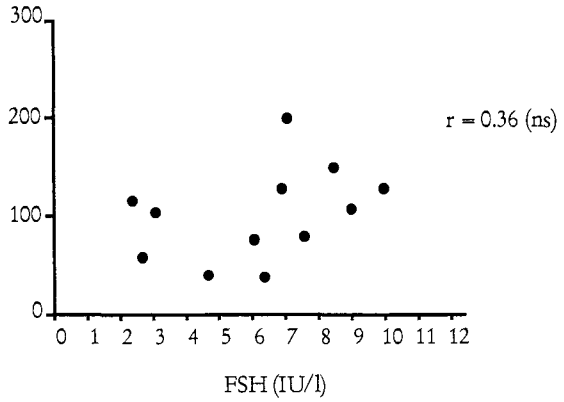
respectively) were significantly different from the normal responders whose mean inhibin-B level was 176 pg/ml (range 127–215).

Inhibin-B also has a role to play in the optimisation of non-analogue IVF, where elevated inhibin-B levels predict patients at risk of a premature LH surge which is associated with the recovery of low numbers of immature oocytes and a reduced pregnancy rate (Lockwood *et al.* 1997b).

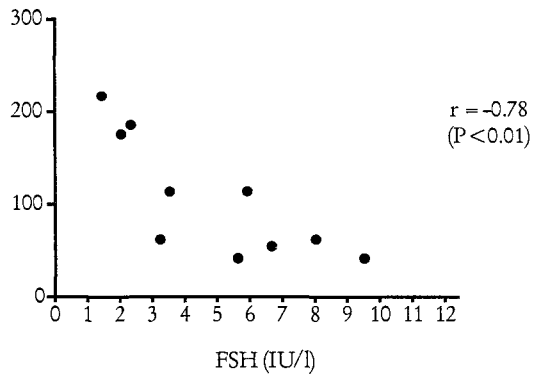
Seifer and co-workers (Seifer *et al.* 1999) have demonstrated that women with declining ovarian reserve show evidence of a decrease in day 3 inhibin-B levels before a rise in day 3 FSH levels. In this study, comparison was made between 109 women with normal ovarian reserve undergoing ART for tubal factor or male factor and 47 women with declining ovarian reserve as proven by poor response to previous gonadotrophin stimulation. There was no difference in gravidity or day 3 serum FSH levels between the two groups, but the women with declining ovarian reserve had significantly lower day 3 inhibin-B levels (80.2 +/– 7.3 vs. 108.1 +/– 6.1 pg/ml), produced fewer oocytes, had a higher cancellation rate and required higher levels of gonadotrophin stimulation.

The study by Hofmann *et al.* (Hofmann *et al.* 1998), investigated a correlation between inhibin-B levels and results of a clomiphene citrate challenge test (CCCT) in 19 patients with normal ovarian reserve testing and in 15 whose CCCT was abnormal. Inhibin-B levels were higher on day 10 than day 3 for all patients, and women with normal CCCT results had higher inhibin-B levels on both days than those with diminished ovarian reserve. There was a negative correlation between FSH and inhibin-B levels on day 3 and day 10 and a positive correlation was found between day 10 E_2 and inhibin-B. Lockwood *et al.* (1998) have reported a significant negative correlation between mid-follicular phase inhibin-B and FSH levels in fertile women and women undergoing IVF for tubal factor infertility, but no such negative correlation in women with a diagnosis of "unexplained" or mild male factor infertility (Fig. 8.8).

In a prospective study of older fertility patients (aged 39–44 years) undergoing a first cycle of IVF, Lockwood *et al.* (Lockwood *et al.* 1998b) found that low inhibin-B levels were more prognostic of reduced ovarian reserve and imminence of the perimenopausal transition than age, cycle



(a)



(b)

Fig. 8.8 (a) Correlation between follicular phase FSH and inhibin-B in patients with unexplained infertility (n = 12). **(b)** Correlation between follicular phase FSH and inhibin-B levels in normal, fertile women (n = 10) (data from Lockwood *et al.* 1998, with permission).

characteristics or basal FSH levels. In this study, 35 fertility patients with regular menstrual cycles undergoing a first IVF treatment had blood samples taken in two consecutive cycles. Fourteen of the women had FSH levels >10 IU/L at one of the FSH estimations, and nine were >10 IU/L at

both. In all patients, including those with varying FSH levels, inhibin-B levels were constant between cycles (between cycle variation <10%). Mean inhibin-B levels were highest in patients with normal FSH levels and consistently lower in patients with raised or variable FSH levels. At oocyte pick-up, inhibin-B was significantly positively correlated with follicle number and oocyte number. Six patients with very low inhibin-B levels (<45 pg/ml) developed menstrual irregularity and persistently raised FSH levels within six months of treatment.

In a study to assess whether measuring inhibin-B levels would increase the sensitivity of traditional ovarian reserve testing, Morris *et al.* (Morris *et al.* 1998) evaluated 47 infertile and six menopausal women. The mean day 3 inhibin-B level in the infertility group was 54 pg/ml (range 0–230) and the infertility patients with a raised FSH (>10 IU/L) had a mean inhibin-B of 20.5 pg/ml (range 0–38). They concluded that a low inhibin-B, notwithstanding a normal FSH, E_2 and CCCT, was a prognostic factor for poor outcome with ART.

The results of these studies have important implications for the counselling and management of fertility patients, and they emphasise the interdependence of FSH, inhibin and dynamic tests of ovarian reserve. Many studies demonstrate the expected negative correlation between inhibin-B and FSH, even in fertile women, and these data are consistent with the hypothesis that a "mass-effect" decline in circulating inhibin-B, as the number of primary follicles within the ageing ovary falls, may precede and precipitate the early follicular phase rise in FSH seen during the perimenopausal transition. The absence of this negative correlation in infertility patients with "unexplained" or mild male factor infertility may suggest that these patients have a degree of ovulatory impairment (Lockwood *et al.* 1998b) It may be that some cases of previously "unexplained" infertility might be explained by the female partner having a reduced ovarian pool of primordial follicles with resultant adverse effect on follicle cohort size, oocyte quality and fertility despite regular ovulation and normal basal gonadotrophin levels. It is recognised that, even if women who respond poorly to a standard drug regimen subsequently demonstrate a reasonable response to a much higher dose of stimulation, they still have a significantly poorer prognosis for pregnancy (Jenkins *et al.* 1991, Van Rysselberge *et al.* 1989).

In studies of older women undergoing a first cycle of IVF, the finding of a high level of inter-cycle variation in serum FSH in women with a single raised FSH result, implies that an isolated FSH level, irrespective of whether it is above the normal range, is an unreliable guide to likely ovarian response. Even within the normal range of FSH, there appears to be a trend towards a poorer response with increasing serum FSH level (Cahill *et al.* 1994). Significant inter-cycle variations in FSH limit the usefulness of a single measurement (Scott *et al.* 1990) and even if the FSH level is normal or low, it has been suggested that an elevated FSH to LH ratio may indicate a poor response (Mukherjee *et al.* 1996). However, a recent study by van Santbrink (van Santbrink *et al.* 1995) found up to 2.5-fold differences in maximum serum FSH levels in a well-defined group of young women with normal ovarian function, and concluded that high FSH concentrations may not necessarily indicate decreased ovarian reserve.

The low level of intercycle variation in inhibin-B levels seen even where follicular phase FSH levels were highly variable suggests that inhibin-B is providing a more "direct" measure of the mass of follicles potentially available for recruitment (Fig. 8.9). FSH levels are co-modulated by factors other than inhibin including oestrogen and other growth factors (Seifer *et al.*

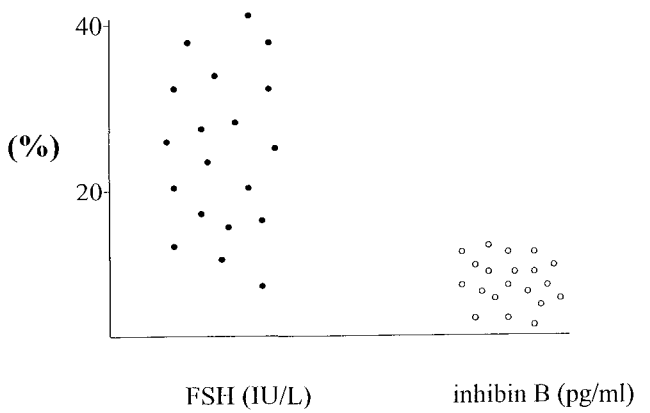


Fig. 8.9 Intercycle variation in FSH and inhibin-B in fertility patients aged >35 years. (data from Lockwood 1999a, with permission).

1995) and therefore may provide an indirect measure of response to endogenous FSH (which is only partially representative of how the ovary may respond to exogenous FSH).

The "reversed" FSH:LH ratio seen in poor responders may itself be a reflection of low inhibin-B levels as LH is being suppressed by the usual gonadotrophin modulators, but FSH is not being suppressed by the low circulating levels of inhibin-B. These studies also identified several patients who had high/variable FSH levels but nevertheless responded well to ovulation induction. In all cases, they had discordant (i.e. inappropriately high) inhibin-B levels and this finding offers further support for a possible role for inhibin-B as a marker of ovarian reserve.

8.7 Inhibin and Activin in Early Pregnancy

Serum concentrations of immunoreactive (ir) inhibin has been reported to be higher during various stages of pregnancy compared with non-pregnant subjects (McLachlan *et al.* 1987, Abe *et al.* 1990, Tabie *et al.* 1991, Yohkaichiya *et al.* 1991, Baird & Smith 1993, Tovanabutra *et al.* 1993). In all of these previous studies ir-inhibin was measured using radio immuno-assays (RIAs) which cross-react extensively with different inhibin α-subunit forms. Consequently, the contribution of the biologically active dimeric inhibin forms was unknown. The development of specific and sensitive assays for dimeric inhibins has facilitated the measurement of these proteins throughout human pregnancy (Muttukrishna *et al*, 1995). In human pregnancy, inhibin-A is the major circulating form of inhibin with levels of inhibin-B near the detection limit of the assay (Muttukrishna; unpublished observation). Serial changes in the concentrations of inhibins A and B during the establishment of pregnancy have been reported by Illingworth *et al.* (1996). In spontaneous singleton pregnancies, inhibin-A levels start rising markedly from five weeks to reach a peak concentration at eight weeks gestation. After eight weeks, inhibin-A levels start to decline up to 11 weeks, consistent with the pattern reported in the cross-sectional study (Muttukrishna *et al.* 1995; see Chapter 5, Fig. 5.5). Inhibin-B levels did not rise in early pregnancy and levels were near the detection limit of the assay (10 pg/ml).

Measurement of activins in circulation has been difficult due to the presence of the high-affinity activin-binding protein follistatin. However, the measurement of "total" (bound activin + free activin) in circulation was made possible by the development of a sensitive, specific and accurate enzyme immunoassay (EIA) (Knight *et al.* 1996). Using this assay, circulating concentrations of "total" activin-A have been measured throughout pregnancy. (Muttukrishna *et al.* 1996). During pregnancy, by eight weeks gestation serum, activin-A concentrations were higher than those during the normal menstrual cycle (see Chapter 5, Fig. 5.5). Levels of activin-A did not vary significantly during the remainder of the first and second trimesters. After 24 weeks, serum activin-A levels rise with a marked increase at term.

In our recent study (Lockwood *et al.* 1997c), the source of inhibins in early pregnancy was investigated by comparing inhibin-A and activin-A profiles in pregnancies conceived *in vitro* with or without corpus luteum function. This study demonstrated that the elevation of circulating levels of dimeric inhibin-A in early pregnancy is the result of production by the feto-placental unit, since comparable levels of inhibin-A were found in singleton pregnancies arising from spontaneous conceptions and following IVF treatment with both fresh and frozen embryo transfer. In the first case, a single corpus luteum would be expected, in the second case, multiple corpora lutea are routinely observed, and in pituitary down-regulated frozen embryo replacement (FER) cycles no corpus luteum is present (Fig. 8.10). The significantly higher levels of inhibin-A found in multiple pregnancies is further evidence for this. The very low levels of inhibin-A found in pregnancies that became missed abortions and the undetectable levels seen in "biochemical" pregnancies, notwithstanding their "normal" hCG levels, is further evidence for this hypothesis and reflects the short half-life of inhibin-A in the circulation as previously demonstrated by Muttukrishna *et al.* (1997a).

The significant differences in levels of inhibin-A in viable, non-viable and multiple IVF pregnancies detectable as early as 13 days following embryo transfer (ET) is further evidence as to the source of inhibin-A in early pregnancy and suggests that estimation of inhibin-A may be helpful in the management of early pregnancy in IVF patients especially in the presence of bleeding or symptoms of OHSS. Early diagnosis of a complicated or poor pregnancy outcome could aid the counselling and management of this group of fertility patients.

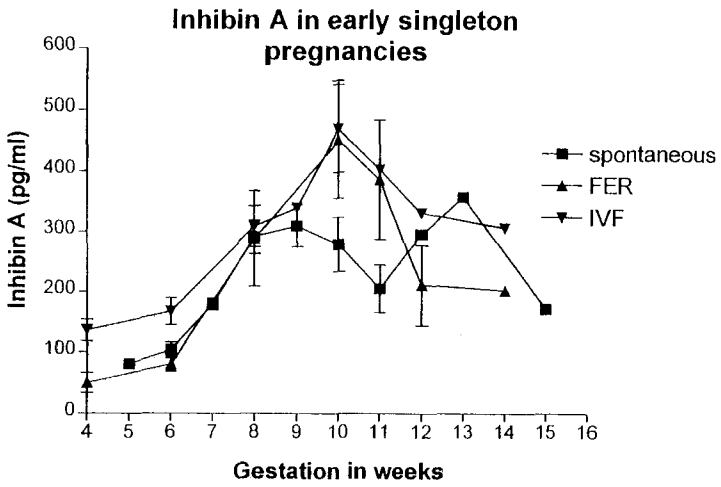


Fig. 8.10 Concentrations of inhibin-A at 4–14 weeks gestation in spontaneous singleton pregnancies and singleton IVF pregnancies with fresh (IVF) and frozen embryo transfer. Values are mean ± SEM (data from Lockwood *et al.* 1997c, with permission).

Levels of pro-α_C containing inhibins are significantly higher in multiple IVF pregnancies compared with IVF singletons and in IVF singleton conceptions with fresh ET compared with FER conceptions and spontaneous conceptions (Fig. 8.11). In spontaneous and IVF singletons, the levels of pro-α_C were seen to decline as the pregnancies progressed, reflecting waning corpus luteum function. The identification of the corpora lutea as the principle source of pro-α_C in pregnancy is possibly highly significant for understanding the development of OHSS.

8.8 Inhibin Pro-α_C and the Prediction of Ovarian Hyperstimulation Syndrome (OHSS) in IVF Treatment

OHSS is a severe, potentially life-threatening complication of gonadotrophin-stimulated ART treatment cycles. The incidence of significant OHSS-complicating assisted conception is variably quoted as between 0.6% and

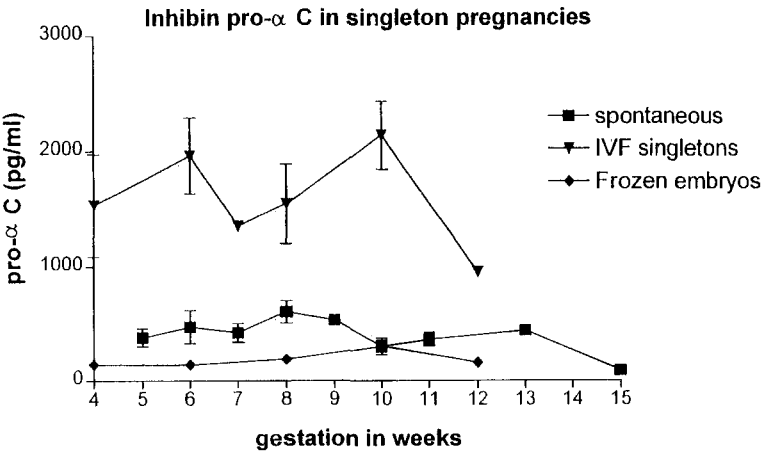


Fig. 8.11 Serum concentrations of inhibin pro-α_C containing inhibins in spontaneous singleton pregnancies and singleton IVF pregnancies with fresh (IVF) and frozen embryo replacement (data from Lockwood *et al.* 1997c, with permission).

14%. The syndrome is associated with excessive follicular development and elevated serum levels of oestradiol. The severity of the symptoms, however, is often poorly correlated with these markers and, unless pregnancy occurs, the syndrome is usually self-limiting. The underlying cause of OHSS is unknown, but a vasoactive ovarian factor such as vascular endothelial growth factor (VEGF) is likely to be involved. Following the demonstration that inhibin pro-α_C is principally a product of the corpus luteum of pregnancy [since it is found in very low levels in pregnancies conceived using frozen-thawed embryos and in high levels in multiple pregnancies (see Fig. 8.11)], it was hypothesised that elevated levels of inhibin pro-α_C in the luteal phase of an ART treatment cycle may be associated with an increased incidence of OHSS.

In a prospective study (Lockwood *et al.* 1998c), serum was collected from women who presented with significant symptoms of OHSS following embryo transfer and prior to their pregnancy test, and from a control group of women undergoing IVF-ET without symptoms. Significant symptoms

included abdominal swelling, nausea and breathlessness. All women in the study had at least two and a maximum of three embryos transferred. The serum from 12 women who conceived during that treatment cycle and developed mild to moderate OHSS was assayed for inhibin pro-α_C, progesterone and oestradiol. The serum results were compared with non-conception cycles and cycles in which pregnancy occurred but OHSS did not develop. The average inhibin pro-α_C level in women who conceived and developed moderate OHSS was 5256 ± 798 pg/ml ($n = 12$) compared to 2671 ± 366 pg/ml in the non-pregnant women with OHSS ($n = 8$) and 1889 ± 267 in pregnant women without symptoms of OHSS ($n = 24$). ($p < 0.05$). (There was a strong correlation between oestradiol levels and pro-α_C on the day of oocyte retrieval ($r = 0.67$ $p < 0.01$), but this correlation was not present in the late luteal phase.

It was concluded that oestradiol and progesterone levels in the luteal phase are a relatively poor indicator of the risk of developing OHSS or predicting when conception is likely to occur. The combination of elevated pro-α_C levels and symptoms of OHSS is highly predictive of pregnancy and hence the probable prolongation and exacerbation of symptoms of the syndrome. The management of the patient with OHSS can be appropriately influenced by fore-knowledge of whether the patient is pregnant and hence whether the symptoms are likely to be self-limiting. Given the iatrogenic nature of OHSS, it remains particularly important for the clinician to minimise the risk of OHSS in women receiving fertility treatment and to recognise situations in which it is developing as soon as possible.

8.9 Inhibin and Activin and the Prediction of Pre-Term Labour

Pre-term labour remains one of the most significant causes of neonatal and perinatal morbidity and mortality. Multiple pregnancies and maternal syndromes such as intra-uterine infection and pre-eclampsia are heavily implicated in the occurrence of pre-term labour. Developments in neonatal intensive care such as the introduction of artificial surfactant and more physiological ventilation regimes have increased the incidence of intact

survival for very premature and low birth weight babies. Numerous endocrine factors have been investigated as biological markers of pre-term labour including hormones and cytokines such as oestriol, activin, corticotrophin-releasing hormone, interleukin-6 and relaxin.

Petraglia and co-workers (Petraglia *et al.* 1997) reported a study designed to evaluate whether spontaneous labour at term and pathological pre-term labour are associated with changes in the expession of activin-A and activin receptor mRNAs in foetal membranes. They also examined amniotic fluid activin-A concentrations in term and pre-term labours. Activin-β_A subunit and activin receptor type-IIB mRNA levels were significantly higher in both the chorion and amnion of women delivering at term or after pre-term labour compared to women not in labour delivered by elective section. Patients in pre-term labour had significantly higher amniotic fluid activin-A concentrations than women at the same stage of gestation, with the highest levels being found in women with pre-term labour who subsequently delivered. They concluded that spontaneous labour and pre-term labour are characterised by increased synthesis and release of activin-A from amniotic and chorionic cells and by an augmented expression of the activin type-IIB receptor.

Gallinelli *et al.* (1996) identified secretory pulses of activin-A in a cross-sectional study of pregnant women and found that mean values of activin-A were significantly higher in women with pre-term labour than in controls. In a study to assess the extent by which activin-A and follistatin are dynamically regulated during pregnancy, Woodruff *et al.* (1997) found that immunodetectable activin-A is present in the third trimester of pregnant women who have normal onset labour. Follistatin-activin complexes are up-regulated during the third trimester and activin-A production exceeds the binding capacity of circulating follistatin at this time. The implication of this is that activin-A functions as an endocrine factor during the last trimester of human pregnancy and may be involved in the onset of labour.

In a study of chorionic gonadotrophin production and aromatase activity in cultured human placental trophoblasts, Song *et al.* (1996) examined the effect of transforming growth factor-β (TGF-β) and activin-A. They demonstrated that activin-A stimulated hCG production and aromatase activity and speculated that activin may have a role in regulating placental function and the timing and progression of labour.

A single medical development that is of proven benefit to infants born extremely premature is the administration to the mother of high dose steroids which cross the placenta and mature the foetal lungs, but this requires advance warning that labour is impending or may be able to be briefly delayed by the administration of tocolytics. Further work is being undertaken to identify which members of the inhibin-activin family may be able to provide this "early warning".

8.10 Inhibin in Abnormal Pregnancy: Pre-Eclampsia

Pre-eclampsia is a potentially severe maternal syndrome associated with placental dysfunction of unknown aetiology, the onset and progress of which is unpredictable (Redman 1991). Muttukrishna *et al.* reported that inhibin-A and activin-A levels are significantly raised in pre-eclampsia (defined as sustained diastolic pressure ≥ 90 mmHg from previously lower levels and sustained proteinuria ≥ 0.3 mg/24h) compared to gestational age matched controls. All patients reported in this study developed pre-eclampsia around 30 weeks gestation (Muttukrishna *et al.* 1997b). As shown in Fig. 8.12, there was no overlap in the levels of inhibin-A and activin-A in pre-eclampsia cases and the controls (with a "cut-off" value of 1 ng/ml for inhibin-A and 10 ng/ml for activin-A). Studies carried out in early pregnancy (15–18 weeks gestation) have shown a significant rise ($\sim 60\%$) in inhibin-A concentrations in pregnant women who developed pre-eclampsia later in pregnancy compared to normal pregnant women (Muttukrishna *et al.* 1997c). A study comparing median levels of maternal serum inhibin-A in second trimester blood samples in 28 pregnancies which developed pre-eclampsia compared with 701 matched controls found that median inhibin-A levels were 2.01 multiples of the gestational-specific median for the controls (Cuckle *et al.* 1998). In this study, the predictive samples were taken, on average, 22 weeks before the diagnosis of pre-eclampsia was made. Based on these observations, it could be speculated that women with raised inhibin-A levels at 16 weeks gestation (in a pregnancy unaffected with Down's syndrome) are at a higher risk of developing pre-eclampsia.

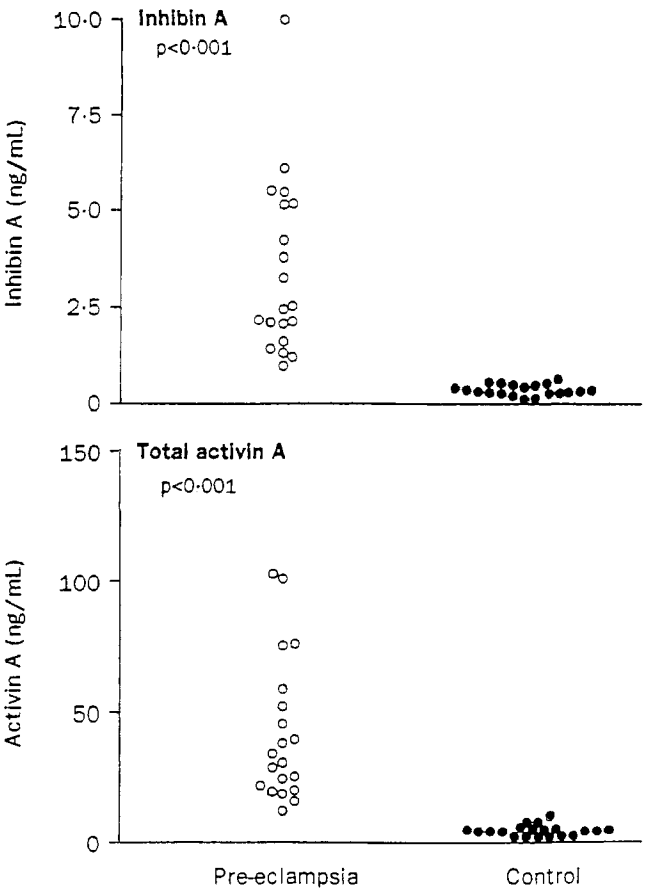


Fig. 8.12 Scatter plot of individual serum concentrations of inhibin-A and activin-A in normal controls and women who developed pre-eclampsia (data from Muttukrishna *et al.* 1997a, with permission).

Since early intervention may allow the development of the syndrome to be delayed or ameliorated and the neonatal prognosis can be enhanced by specific medical management, the ability to predict which pregnancies may become affected by pre-eclampsia holds great promise for the management of this disease and may provide an opportunity to study the early natural history of the disease and conduct treatment trials. The elevation of activin-A

in pre-eclampsia affected pregnancies noted by Muttukrishna *et al.* was also reported by Petraglia and co-workers (1995), and they found that pregnant women with chronic hypertension and women with pregnancy-associated hypertension did not show the significantly elevated levels seen in women with frank pre-eclampsia.

8.11 Inhibin in Screening for Down's Syndrome

Antenatal screening for causes of severe handicap, such as trisomy-21 (Down's Syndrome), has become an established part of obstetric practice. Although biophysical markers such as detection of increased nuchal fold thickness using ultrasound (Szabo *et al.* 1990, Nicolaides *et al.* 1994) have been adopted in some centres, serum screening remains the mainstay of antenatal screening for this condition. Early work focussed on the finding of abnormally low levels of α-fetoprotein (AFP) in sera collected from women carrying a foetus with Down's syndrome (Cuckle *et al.* 1984), but it soon became apparent that other serum markers were also abnormally raised or suppressed in cases of trisomy-21, and that these might usefully be combined into screening tests with acceptable sensitivity and specificity.

Three studies using the inhibin α-subunit directed assays (Van Lith *et al.* 1992, Spencer *et al.* 1993, Cuckle *et al.* 1994), and seven studies using the dimeric inhibin-A assays have shown that measuring maternal serum inhibin-A in second trimester could contribute to the existing screening tests (Cuckle *et al.* 1995, Canick *et al.* 1994, Aitken *et al.* 1996, Wallace *et al.* 1996, Wenstrom *et al.* 1997, D'Antona *et al.* 1998, Haddow *et al.* 1998). Down's screening results were 77% predictive if inhibin-A was added as a fourth marker to the existing triple test (AFP, unconjugated estriol (UE3), hCG-β and age) from 15–18 weeks gestation. An advantage of the use of inhibin-A is the very small change in average inhibin-A from 15–18 weeks gestation.

Levels of inhibin-A in amniotic fluid collected from normal pregnancies and those affected by Down's syndrome have also been investigated (Riley *et al.* 1997, Wallace *et al.* 1997). Levels were significantly lower in Down's pregnancies, suggesting a possible origin for inhibin-A from the foetal membranes in the second trimester and possible clinical efficacy as a rapid

test for the presence of a Down's foetus by assay of inhibin species in amniotic fluid. A recent report (Muttukrishna *et al.* 1999) which analysed 146 amniotic fluid samples obtained at amniocentesis (15–20 weeks gestation) showed that amniotic fluid concentrations of inhibin-A, inhibin-B and activin-A gradually increase with gestation while concentrations of follistatin are similar between weeks 15 and 20.

There is emerging evidence that serum screening for dimeric inhibin-A may also be helpful in the detection of aneuploidies other than Down's Syndrome at a rate superior to that of the traditional analyte combination (Wenstrom *et al.* 1998). Developing serum screening in twin pregnancies has been complicated, but a nested case-control study by Watt *et al.* (1996) found that the median inhibin-A level in the twin pregnancies studied ($n = 200$) was 1.99 MOM value for 600 gestation matched singleton pregnancies ($p < 0.001$), and these results enable inhibin-A values to be adjusted so that prenatal screening for Down's Syndrome can be used in twin gestations. Serum screening before 15 weeks of pregnancy has been largely unreliable in this regard, although there are few studies with sufficient power to identify the true potential of a range of markers in combination.

Wald *et al.* (1996) studied a panel of seven markers, including dimeric inhibin-A, in samples collected between eight and 14 completed weeks of gestation, but failed to demonstrate sufficient elevation of inhibin-A in affected pregnancies (median (MoM) 1.19) for it to form a useful adjunct to measurement of free-β hCG and pregnancy associated placental protein (PAPP-A) in the first trimester. Similarly poor discrimination between affected and normal pregnancies was also shown in a second large series (Noble *et al.* 1996), although conversely, Wallace *et al.* (1995) found a more substantial elevation in inhibin-A in first trimester trisomy-21 pregnancies, to 2.46 median MoM (95% confidence interval (CI) 2.11–3.26) in a smaller series of cases. However, levels of inhibin-A are closely correlated with free-β hCG in early pregnancy, suggesting that little would be gained by assay of both markers in a screening panel for trisomy-21 in the first trimester (Noble *et al.* 1996). A clinically useful first trimester serum screen for Down's syndrome remains elusive, and it seems unlikely that measurement of inhibin will be useful in this application. Nevertheless, screening for Down's

syndrome has been significantly improved by the application of inhibin-A assay in the second trimester, illustrating the potential usefulness for inhibin measurements in routine clinical practice.

Summary

- Assessment of serum levels of inhibin-B appears to offer useful prognostic information about ovulatory function and predictive information about response to treatment.
- During very early pregnancy, especially in the presence of complications associated with ART such as multiple gestation and OHSS, measurement of maternal levels of inhibin-A and pro-α_C offers a non-invasive test that can aid the counselling and management of patients.
- In the second trimester, assessment of maternal inhibin-A levels has been shown to improve the sensitivity of the existing serum screening tests for aneuploid pregnancies and may prove clinically useful in the early detection of pregnancies susceptible to developing pre-eclampsia.
- Further research with large numbers of patients and controls needs to be carried out before application to routine clinical practice can be advocated.

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CHAPTER 9

CLINICAL UTILITY OF INHIBIN/ACTIVIN SUBUNITS IN THE IMMUNOPATHOLOGY OF GONADAL TISSUE

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9.1 Introduction

When one considers new entities in gynaecological pathology in recent years, iatrogenic diseases, such as with Tamoxifen or with hormonal replacement therapy, come to mind as one major issue. The other equally important issue is inhibin physiology and its immunohistochemical utility in the pathology of various lesions in the female genital tract, particularly of ovarian tumours. The advent of inhibin in the last few years is, to my mind, the single most significant advance in the practice of gynaecological pathology. This is reflected by the burgeoning literature on this subject, particularly in the last three years. Not only are there variations on the same theme studied, such as the expression of inhibin/activin subunits in sex-cord stromal tumours of the ovary, but there have also been an increasing number of studies showing the utility of these subunits in other gynaecological lesions, and in some cases, in lesions away from the genital tract.

Inhibin/activin subunits have been available for some time now, but were not initially considered for diagnostic experiments in any major way until the evidence of inhibin physiology in various pathological conditions became compelling. For instance, since inhibin serology has been shown to

be an important diagnostic and monitoring tool in ovarian granulosa cell tumours, so has the immunohistochemical use of inhibin/activin subunits in such lesions gained an important place in gynaecological pathology practice. It is indeed the physiology of inhibin gathering momentum which appears to have driven the research in inhibin immunopathology leading to its ever more established role in the examination of gynaecological diseases.

It was as long ago as 1989 when it was first shown that serum inhibin concentrations were raised in women with ovarian granulosa cell tumours (Lappohn *et al.* 1989). Four years later it was recognised that similar elevated serum inhibin levels occurred with mucinous (borderline and invasive) tumours (Healy *et al.* 1993). However, it was only in 1995 that inhibin/activin subunits were first shown immunohistochemically in frozen sections of ovarian tumours (Gurusinghe *et al.* 1995), although the method of raising antisera against the α, β_A and β_B subunits had been described much earlier (Vaughan *et al.* 1989).

Since then, there have been several studies on the same theme, particularly in showing inhibin/activin subunit immunoreactivity in formalin-fixed, paraffin-embedded sections of ovarian tumours, particularly in the sex cord-stromal group (Burger *et al.* 1996, Arora *et al.* 1997, Flemming P. 1996, Zheng *et al.* 1998, McCluggage *et al.* 1997, Costa *et al.* 1997, Hildebrandt *et al.* 1997, Zheng *et al.* 1997, Stewart *et al.* 1996, Pelkey *et al.* 1998, McCluggage *et al.* 1998, Guerrieri *et al.* 1998).

More recently, the clinical utility of inhibin/activin subunit immunopathology has been expanded to cover other lesions of the ovary and other tissues in the genital tract such as the placenta (McCluggage *et al.* 1998). Its utility in gestational trophoblastic disease (Shih & Kurman 1999) and in placenta from cases of pre-eclamptic toxaemia (Jackson *et al.* 2000) has been demonstrated. The usefulness of inhibin immunocytochemistry in ovarian cyst aspiration cytology (McCluggage *et al.* 1998, Manek & Charles 1998) has also been shown and should make a major impact in this science. With the increasing knowledge of inhibin/activin physiology in polycystic ovarian syndrome (PCOS), it was inevitable that the PCOS ovary needed examination by inhibin/activin immunohistochemistry (Lockwood *et al.* 1999).

Localisation of inhibin and/or activin in other tissues such as the testis (Moore *et al.* 1994, Kommoss *et al.* 2000), epididymis (Phadke *et al.* 1991), prostate (Gade & Sheth 1989), osteoclasts (Hosoi *et al.* 1996), and more

recently in the adrenal cortex (McCluggage *et al.* 1998, Munro *et al.* 1999) has also been demonstrated.

To date, there have been virtually no immunohistochemical studies with follistatin in gonadal tissue. Only one study has shown immunohistochemical localisation of activin-A and follistatin in various tissues such as the ovary, testis, pancreatic islets, pituitary gland, thyroid follicles and adrenocortical cells (Wada *et al.* 1996).

Some of the studies mentioned above will now be discussed in slightly greater detail under common subheadings to evaluate what work has been done with inhibin/activin subunit immunopathology in the recent years.

9.2 Methodology

Most of the immunohistochemical studies have utilised the two commercially available monoclonal antibodies (Serotec, Oxford, UK). These are to the α-subunit (inhibin) and to the β_A-subunit (inhibin-A or activin-A). These antibodies can be used on formalin-fixed, paraffin-embedded sections and the immunoperoxidase technique is employed. This involves application of the antibody followed by incubation with a peroxidase conjugated streptavidin biotin complex. The reaction product is detected with 2, 3-diaminobenzidine and counterstained with haematoxylin. The whole procedure is well described in most of the studies (Arora *et al.* 1997, Shih *et al.* 1999). The β_B-subunit is available for use on frozen sections (Gurusinghe *et al.* 1995).

In cytology, the immunoperoxidase technique can be used, provided the indigenous biotin of the ovarian stroma is blocked, which is easier to achieve in histological sections. The alkaline phosphatase-anti alkaline phosphatase (APAAP) technique is the method of choice in cytological preparations particularly because the cells are not generally fixed, either in alcohol or formalin.

9.3 Ovarian Tumours

It is in ovarian tumours, particularly the sex cord-stromal tumour group, that inhibin/activin immunohistochemistry has been most extensively studied.

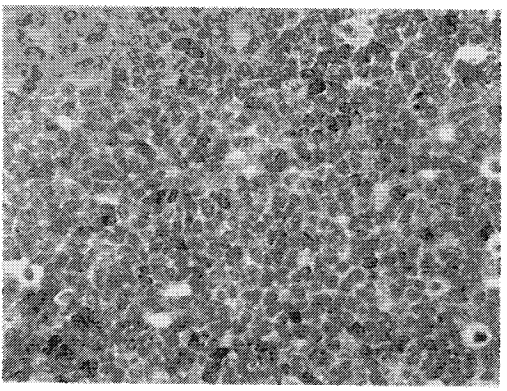

Fig. 9.1 α-inhibin subunit staining of moderate intensity in an ovarian granulosa cell tumour.

This probably reflects the discovery of raised serum inhibin levels in women with ovarian tumours (Healy *et al.* 1993), especially granulosa cell tumours (Lappohn *et al.* 1989). Most studies of varying sizes have shown that inhibin (α and β_A subunits) is strongly expressed in granulosa cell tumours (Arora *et al.* 1997, Gurusinghe *et al.* 1995, Choi *et al.* 2000) (Fig. 9.1).

It has been suggested by several authors that this staining property could be successfully used to make a diagnosis of a sex cord-stromal tumour when morphologically there may be other tumoxurs in the differential diagnosis (McCluggage *et al.* 1997, Costa *et al.* 1997, Hildebrandt *et al.* 1997). Hence it could be used to differentiate granulosa cell tumours from primary or metastatic small cell carcinomas in the ovary, from lymphomas, from other poorly differentiated epithelial tumours (such as endometrioid carcinomas), from germ cell tumours, and from other miscellaneous tumours. The antibody to the α-subunit of inhibin has been found to stain focally and weakly in a substantial number of other primary and metastatic poorly differentiated carcinomas in the ovary (Hildebrandt *et al.* 1997, Choi *et al.* 2000, Zheng *et al.* 2000). This is thought to be in luteinised stromal cells surrounding or admixed with the neoplasm and hence needs cautious interpretation. However, inhibin/activin subunit immunore-activity can be utilised as part of a panel of immunohistochemical stains to reliably differentiate sex cord-stromal

tumours from other tumours which are morphologically similar. In particular, the use of cytokeratins 7 (Guerrieri *et al.* 1998) and 20, CA-125 and epithelial membrane antigen are the most useful adjunct immunostains to use in a panel (personal observations).

Some of the aforementioned studies have shown immunopositivity with inhibin/activin subunits, particularly with the α-inhibin antibody in sex cord-stromal tumours other than granulosa cell tumours (Hildebrandt *et al.* 1997, McCluggage *et al.* 1998). These include fibrothecomas and steroid cell tumours (Costa *et al.* 1997, Hildebrandt *et al.* 1997). Inhibin immunohistochemistry would therefore not be a useful diagnostic tool in differentiating the various sex cord-stromal tumours but the morphological features on routine stains of each of the main entities normally allow a diagnosis without the need for immunohistochemistry. Inhibin immunoreactivity would, however, be useful in detecting recurrances of granulosa cell tumours even in image-guided core biopsies where the classical architecture may not be apparent (personal observations).

An interesting finding has been the demonstration of the β_A-subunit (inhibin/activin) expression in cells of epithelial tumours (Zheng *et al.* 1998), particularly in mucinous lesions (Arora *et al.* 1997, Choi *et al.* 2000) (Fig. 9.2).

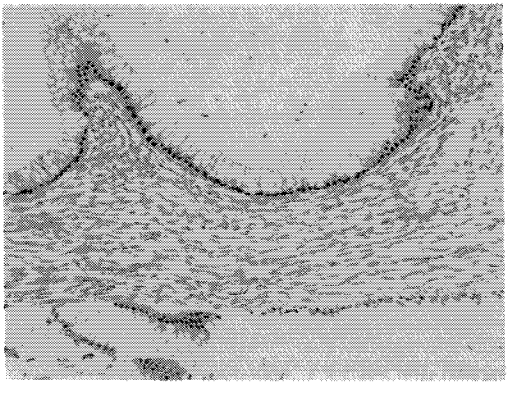

Fig. 9.2 β_A-subunit (activin) positive staining in mucinous cells in an ovarian mucinous tumour.

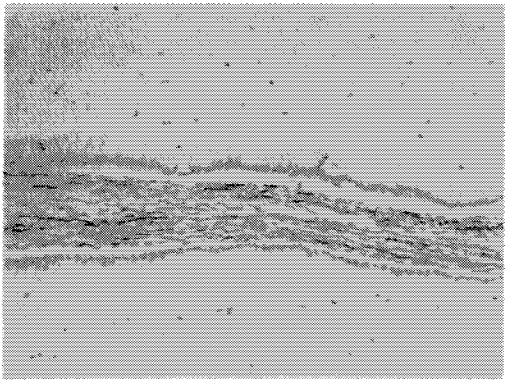

Fig. 9.3 α-inhibin subunit positive staining in stromal cells but negative staining of the mucinous cells in the same neoplasm as shown in Fig. 9.2.

Moreover, whilst the α-inhibin subunit staining has been shown to be negative in the same cells, there is often positivity in cells within the surrounding stroma (Zheng *et al.* 1998, 2000) (Fig. 9.3).

It has been suggested that activin in ovarian epithelial tumours may exert an autocrine and/or paracrine effect resulting in the proliferation of epithelial tumour cells. This is further supported by studies showing activin-stimulated cell proliferation in ovarian cancer cell lines (Di Simone *et al.* 1996, Fukuda *et al.* 1998, Zheng *et al.* 2000). It is possible that activin plays a significant role in the epithelial/stromal interactions within epithelial neoplasms. Recent findings have suggested that the inhibin α-subunit may exert its action as a tumour suppressor gene (Matzuk *et al.* 1994), although this conflicts with the finding of a high immunohistochemical expression of inhibin in some ovarian tumours. One study has correlated inhibin/activin expression in ovarian granulosa cell tumours with survival and shown its role as a prognostic index (Ala-Fossi *et al.* 2000).

9.4 Ovarian Cyst Aspiration Cytology

Ovarian cyst aspiration cytology is not a routine practice in many centres mainly due to limitations in the interpretation of cytological/morphological

features and due to problems in recognition of scanty, distorted cells with routine stains. It is important to differentiate epithelial cells in ovarian cyst fluids from follicular/luteal cells (Fig. 9.4 and 9.5). The presence of epithelial cells indicates a neoplasm which requires removal in case of malignant transformation, while the presence of follicular cells is sufficient for the diagnosis of a functional cyst which need not be removed. The demonstration

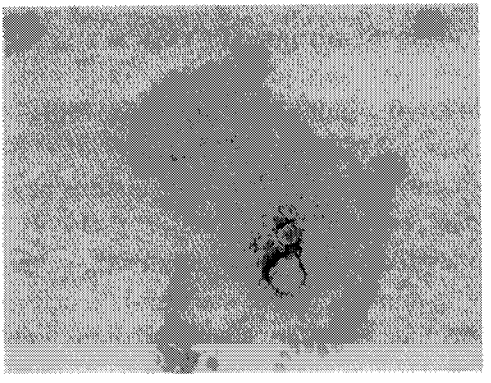

Fig. 9.4 α-subunit positive staining in follicle cells aspirated from a functional ovarian cyst (APAAP technique).

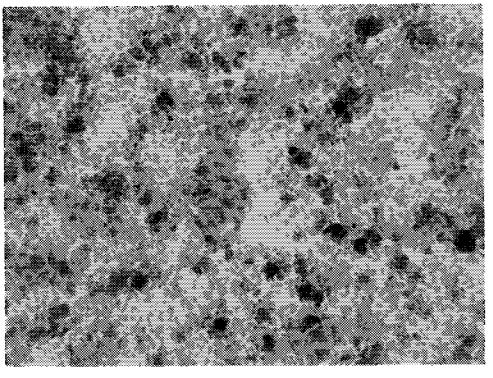

Fig. 9.5 α-inhibin subunit positive staining in luteinised cells aspirated from an haemorrhagic functional ovarian cyst of corpus luteal type (APAAP technique).

of immunocytochemical inhibin α-subunit positivity in cells from an ovarian cyst confirms follicular cells and hence a functional cyst (Fig. 9.4). This has been shown by two studies (McCluggage *et al.* 1998, Manek & Charles 1998). I have shown the utility of inhibin α-subunit antibody staining with a panel of other antibodies including BerEp4 (epithelial cell marker) and CA-125 (Manek & Charles 1998).

9.5 Polycystic Ovarian Syndrome (PCOS)

The physiology involving inhibin/activin and follistatin is reasonably characterised in cases of PCOS (Lockwood *et al.* 1998). Recently, the immunostaining patterns of the α, β_A and β_B subunits have been studied and compared to those in normals (Lockwood *et al.* 1999). Staining with the β_A-subunit is much less dense than that with the β_B-subunit and less than in control ovaries, but α-inhibin subunit staining is much more dense than in control ovaries reflecting the high levels of serum inhibin-B in women suffering from PCOS (Fig. 9.6).

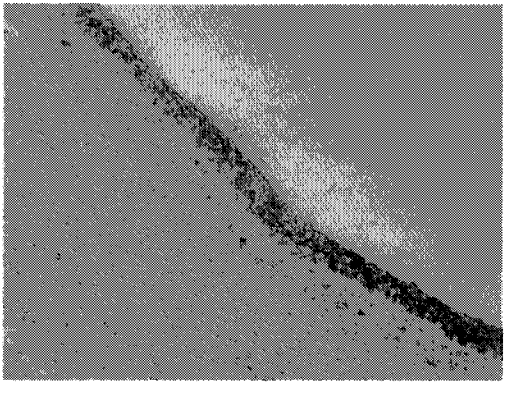

Fig. 9.6 β_A-subunit staining in inner follicular cells and outer theca cells in a cyst from a polycystic ovary (APAAP technique).

9.6 Other Genital Tract Tissues

Inhibin immunoreactivity, particularly with the α-subunit, has been demonstrated in placental tissue (McCluggage *et al.* 1998) and more recently in the placentae of pre-eclamptic toxaemia (Jackson *et al.* 2000) and in gestational trophoblastic diseases (Shih & Kurman 1999). All three studies have shown inhibin expression in syncytiotrophoblasts but not in cytotrophoblasts, and in some cases, within decidua. Inhibin expression may be seen in all trophoblastic lesions, including choriocarcinoma and hence can be used to differentiate these lesions from its mimics (Shih & Kurman 1999). It can also be seen in non-gestational trophoblastic tissue and is useful in picking up trophoblastic cells in germ cell tumours (McCluggage *et al.* 1998). I have shown that the intensity of β_A and α-subunit staining is much greater in the syncytiotrophoblasts of pre-eclamptic placentae compared to controls, and have postulated that the placenta is the source for the raised serum inhibin levels in pre-eclampsia (Fig. 9.7) (Jackson *et al.* 2000).

Immunohistochemical activin-A has been shown in the human endometrium during the various stages of the menstrual cycle and in the decidua of early pregnancy, suggesting that the endometrium is the major source of activin-A during a normal menstrual cycle and the decidua is the source in

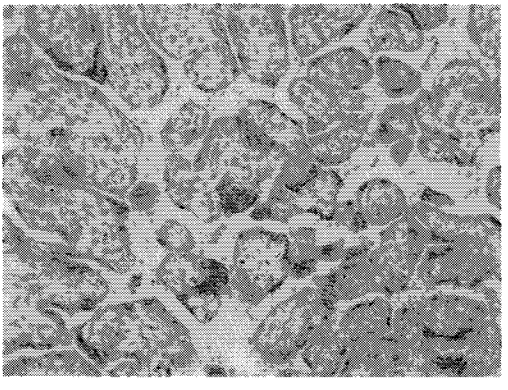

Fig. 9.7 Strong β_A-subunit staining in syncytiotrophoblastic cells in a pre-eclamptic placenta.

early pregnancy (Otani *et al.* 1998). Petraglia *et al.* (1998) have shown the presence of inhibins A and B and activin-A in cell cultures from cervical and endometrial carcinomas. Recently α-inhibin staining was utilised to show true sex cord differentiation in a uterine tumour resembling ovarian sex cord tumours (McCluggage 1999, Baker *et al.* 1999).

Wolffian adnexal tumours in the ovary are often inhibin-α positive (Devouassoux-Shisheboran *et al.* 1999). In contrast, sex cord-like elements in adenomyosis (Fukunaga 2000) and sertoliform areas of ovarian endometrioid carcinomas (Ordi *et al.* 1999) are inhibin negative.

9.7 Other Organs

Immunohistochemical α-inhibin can be seen in Sertoli cells of testicular tubules and Leydig cells of the testicular interstitium (Figs. 9.8 and 9.9) (Moore *et al.* 1994, Kommoss *et al.* 2000, personal observations). Inhibin α-subunit expression can also be seen in Leydig cell tumours (Kommoss *et al.* 2000, personal observations).

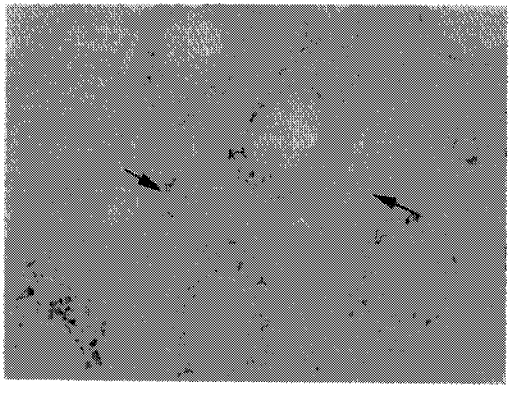

Fig. 9.8 α-inhibin subunit positive staining in Sertoli cells in tubules and Leydig cells in the interstitium (arrow) in a testis.

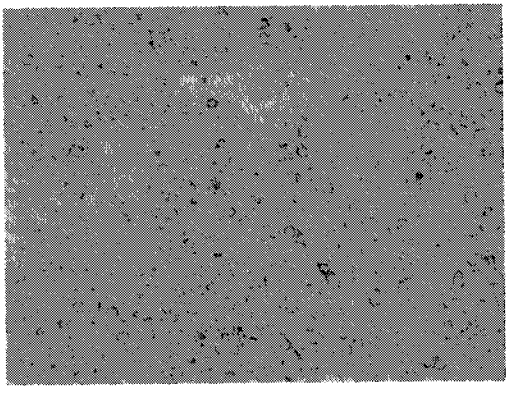

Fig. 9.9 Strong α-inhibin subunit staining in a Leydig cell tumour.

Inhibin/activin subunits have been shown immunohistochemically in other tissues and the number of studies researching this is ever-increasing (McCluggage 1999). At present, it is not in the remit of this chapter to discuss some of these studies, but in the future it may be necessary to do so in the context of the problem of differential diagnoses when several tissues express these subunits.

9.8 Conclusion

Several key studies have been described in this chapter demonstrating the clinical utility of inhibin/activin subunits in the immunopathology of gonadal and extra-gonadal tissue. It is easily recognised that the majority of the studies have been conducted in the past three to four years and many of these have researched tissues away from the genital tract. The clinical utility of inhibin/activin subunit immunopathology in sex cord-stromal tumours, particularly the granulosa cell tumours is well established. Its usefulness in differentiating these tumours from its mimics has also been very well demonstrated. Possible roles for inhibin and activin in tumourigenesis have been postulated in a few studies but the exact

mechanisms of their function and interaction with other factors remain to be determined. With the increasing recognition of many malignancies expressing these subunits, it has been suggested that immunohistochemistry involving inhibin/activin subunits has to be interpreted cautiously and perhaps performed as part of a panel.

The use of inhibin/activin subunit immunopathology has been ably extended to study other ovarian lesions such as polycystic ovaries and has been effectively put to use in ovarian cyst aspiration cytology. Inhibin/activin immunohistochemistry has been successfully utilised in placental and trophoblastic tissue pathology, in the pathology of other female genital tract organs, and in other organs such as the testis and adrenal cortex.

The literature is burgeoning and it is probable that by the time the second edition of this book is being considered, there will be twice as much to consider.

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CHAPTER 10

ACTIVIN RECEPTORS AND THEIR MECHANISM OF ACTION

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10.1 Introduction

Activins are peptide growth and differentiation factors capable of eliciting a wide variety of biological responses from a range of cell types. Originally identified as factors present in ovarian follicular fluid capable of stimulating expression and secretion of follicle-stimulating hormone (FSH) from the anterior pituitary gland, activins have since been purified based on several independent criteria (Vale *et al.* 1990). Functions now ascribed to this ligand family include regulation of hypothalamic, ovarian and placental hormone production, promotion of erythroid differentiation, regulation of cell cycle events, promotion of neural cell survival, inhibition of neural cell differentiation, promotion of bone growth, and involvement in early embryonic development (Bilezikjian & Vale 1992). Strikingly, activin-A is capable of inducing mesodermal tissues from *Xenopus* animal cap explants which otherwise form only ectodermal derivatives (Klein & Melton 1994). In addition, multiple dose-dependent activin responses observed in early embryos have led to the suggestion that activin may act differentially along a gradient of its concentration, as is thought to be the case for classical morphogens (Green *et al.* 1992, Gurdon *et al.* 1994).

Activins are dimeric polypeptides composed of a combination of two structurally related β subunits (β_A and β_B), covalently linked via a single

disulfide bond; the inhibins share this β-subunit, and contain a more distantly related inhibin-specific α-subunit (Vale *et al.* 1990). Although these molecules share a common subunit, the inhibins antagonise the effects of activin in all cell types that respond to both factors. These α and β chains share structural similarity with a host of other growth and differentiation factors which places them in the transforming growth factor β (TGFβ) superfamily of ligands (Kingsley 1994). Additional members of this family include: the bone morphogenetic proteins (BMPs), which promote bone growth among other activities; Müllerian inhibitory substance (MIS), which is involved in male sexual development; decapentaplegic (*dpp*), Vgr60A and *screw*, which are involved in *Drosophila* embryogenesis; the Vg-1 related gene products, which are predicted to regulate early embryonic development in *Xenopus laevis* and other vertebrates; nodal, which is expressed during mouse gastrulation; and dorsalin, which is involved in development of the neural tube.

Because activin regulates such a diverse set of biological processes, and because inhibin antagonises activin functions while sharing a common subunit, a molecular explanation for how these factors signal has been vigorously pursued. A synthesis of biochemical and genetic analyses carried out using systems from *C. elegans* and *Drosophila* to mice and cultured mammalian cells has resulted in an increasingly clear picture of how activin exerts its biological functions. These studies have defined a novel signalling strategy in which activin-regulated transmembrane receptor serine kinases (RSKs) modulate the activity of transcriptional regulators called Smads. The molecular mediators of inhibin function, however, have yet to be identified, although several recent findings suggest that an inhibin receptor may be cloned soon.

10.2 Activin Receptors and the Receptor Serine Kinase Family

High-affinity, activin-specific cell surface binding proteins were identified by binding and chemically cross-linking [^{125}I]-labelled activin-A to multiple cell types. In all cases, two predominant affinity-labelled species were detected corresponding to cell surface proteins with molecular masses of about 50

and 60–70 kDa which have been arbitrarily called type I and type II receptors, respectively (Mathews 1994). Similar observations have been made for TGFβ and other members of the ligand superfamily, suggesting that these factors may share related cell surface receptors. This hypothesis was confirmed as type II receptors for activin (ActRII), and later for TGFβ (TβRII), were cloned using expression cloning techniques designed to exploit the capacity of these molecules to specifically bind radiolabelled ligand (Lin *et al.* 1992, Mathews & Vale 1991). A second type II receptor for activin, ActRIIB, was cloned and shown to exist in multiple alternatively spliced forms (Attisano *et al.* 1992, Mathews *et al.* 1992). ActRII, ActRIIB and TβRII are closely related to the product of the *daf1* gene of *C. elegans* (Georgi *et al.* 1990) and the *punt* gene of *Drosophila* (Childs *et al.* 1993, Letsou *et al.* 1995, Ruberte *et al.* 1995). In addition, seven mammalian type I receptors (ALKs 1–7) (Ebner *et al.* 1993a, Franzén *et al.* 1993, He *et al.* 1993, ten Dijke *et al.* 1993, ten Dijke *et al.* 1994a, Tsuchida *et al.* 1993, Tsuchida *et al.* 1996) for this ligand superfamily have been identified, along with homologs in *C. elegans* (Georgi *et al.* 1990), *Drosophila* (Brummel *et al.* 1994, Nellen *et al.* 1994, Penton *et al.* 1994, Wrana *et al.* 1994b, Xie *et al.* 1994) and *Xenopus* (Graff *et al.* 1994, Suzuki *et al.* 1994). The molecular cloning of these receptors has provided the requisite tools for addressing questions regarding the mechanism of receptor activation and intracellular signalling events.

10.2.1 Type II RSKs

The first milestone in the molecular analysis of activin signalling pathways was the identification and cloning of a type II receptor for activin. Mathews and Vale accomplished this task by using [^{125}I]-labelled activin-A to select for specific binding proteins from a murine corticotropic cell line. Analysis of the deduced amino acid sequence of ActRII revealed a short extracellular domain of 119 amino acids, a single transmembrane spanning region, and an intracellular protein kinase domain with predicted serine/threonine specificity (Mathews & Vale 1991). This observation suggested the existence of a novel cellular signalling strategy, as all previously characterised receptor protein kinases were tyrosine-specific (Ullrich & Schlessinger 1990).

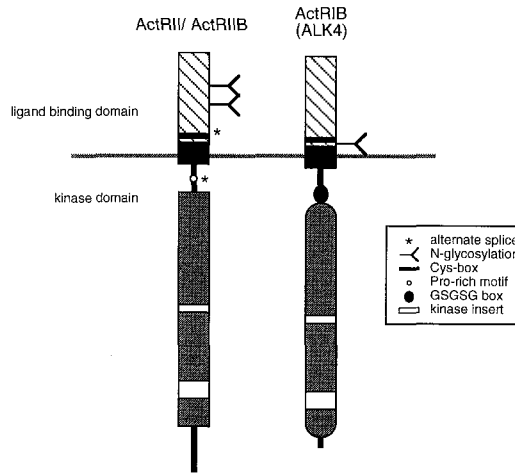

Fig. 10.1 Structural characteristics of the transmembrane receptor protein serine kinases. Schematic representation of type II and type I activin receptors. The extracellular ligand binding domains are hatched, the transmembrane domains are black, and the intracellular kinase domains are grey. Other features are as indicated in the legend.

Subsequently, additional type II receptors for activin (ActRIIB) (Attisano *et al.* 1992, Mathews *et al.* 1992), MIS (Baarends *et al.* 1994, di Clemente *et al.* 1994) and BMPs (Estevez *et al.* 1993, Kawabata *et al.* 1995a, Liu *et al.* 1995, Nohno *et al.* 1995, Rosenzweig *et al.* 1995) were cloned using low-stringency hybridisation, yeast two-hybrid screening, and PCR-based approaches.

Sequence analysis of these molecules indicates that they share characteristic structural features (Fig. 10.1) and belong to a unique family among protein serine/threonine kinases. All members of this receptor family have divergent N-glycosylated extracellular domains of less than 150 amino acids, with only a cysteine-rich motif near the transmembrane region in common. In addition to the conserved intracellular protein serine kinase domain, type II receptors share serine/threonine rich C-terminal extensions of about 20–25 amino acids; a single type II receptor, BMPR-II, has a much longer extension (Kawabata *et al.* 1995a, Liu *et al.* 1995, Nohno *et al.* 1995, Rosenzweig *et al.* 1995). While this extension is phosphorylated *in vivo*

(Souchelnytskyi *et al.* 1996), it is not required for signal transmission (Wieser *et al.* 1993). Both receptor types contain two distinct inserts in the canonical protein kinase domain, the first of which is not required to support signalling through TβRII. However, deletion of the second kinase insert in TβRII abrogates signalling, suggesting that it is required for activity (Wieser *et al.* 1993); additionally, a conserved proline in the second insert was identified as one of the original inactivating mutations of *daf1* (Georgi *et al.* 1990). ActRII and ActRIIB also have proline-rich segments at the juxtamembrane region reminiscent of SH3 recognition motifs which may interact with proteins involved in signal mediatation (Ethier *et al.* 1997).

Initially, the specificity of ligand binding was thought to be wholly dependent on the identity of the type II receptor. Type II activin receptors bind activin-A with a K_d of 100–500 pM, inhibin-A with about ten-fold lower affinity, and do not detectably bind TGFβ (Attisano *et al.* 1992, Mathews & Vale 1991). Similarly, TβRII specifically binds TGFβ with a K_d of 10–50 pM, and cannot be competed with any other ligand family members (Lin *et al.* 1992). These observations indicate that type II receptors participate in specific, high-affinity interactions with their respective ligands. However, we now know that type I receptors can also contribute to the specificity of ligand-receptor interactions. BMP type II receptors, for example, bind ligand poorly by themselves, but form specific high-affinity complexes in the presence of BMP type I receptors (Liu *et al.* 1995, Nohno *et al.* 1995, Rosenzweig *et al.* 1995). Likewise, both ActRII and ActRIIB can bind to BMP2 or BMP7 with low-affinity, but bind with much higher affinity in the presence of type I BMP receptors (Yamashita *et al.* 1995) ActRIIs also appear to mediate BMP signals in *Xenopus* (Chang *et al.* 1996). Therefore, ligand-receptor complexes can assemble in at least two ways.

10.2.2 Type I RSKs

The availability of the type II receptor sequences prompted a rapid search for, and identification of, related RSKs by use of low-stringency hybridisation and PCR cloning strategies. As noted above, additional type II receptors were cloned in this way, but another group of clearly related but distinct

receptor serine kinases was also discovered (Massagué 1998, Mathews 1994). The most complete description of this group of molecules designated them ALKs (activin receptor-like kinase) (Franzén *et al.* 1993, ten Dijke *et al.* 1993, ten Dijke *et al.* 1994a), therefore this nomenclature has been used until a more functional description can be assigned. Although these proteins share homology with type II RSKs, they clearly belong to a distinct family and have different structural features, which now define the type I RSKs (Fig. 10.1). Like the type II RSKs, the extracellular domains are divergent except for conserved spacing of cysteine residues. The intracellular domains contain the conserved serine kinase domain, but lack C-terminal extensions; type I receptors also contain a conserved sequence motif in the juxtamembrane domain consisting of alternating glycine and serine residues (GS domain), which contributes to the activation state of the receptors (see below).

Type I receptors for activin and TGFβ — although normally expressed at the cell surface — do not bind ligand in the absence of type II receptors, based on the results of combinatorial cell culture transfection and binding experiments (Attisano *et al.* 1993, Ebner *et al.* 1993a, Matsuzaki *et al.* 1993, ten Dijke *et al.* 1994a, Tsuchida *et al.* 1993). Initially, the type I molecules were thought to be orphan receptors because none of the known TGFβ superfamily ligands would bind to any of them. This issue was resolved by experiments in which type I and type II receptors were co-expressed in cultured cells and affinity-labelled using [^{125}I] ligand. In the presence of type II receptor, but not when expressed alone, type I molecules become affinity-labelled, indicating some capacity for ligand association (Ebner *et al.* 1993a). In this transient over-expression system, all type I receptors except ALK3 become labelled in the presence of ActRII and activin-A, and all of the ALKs are labelled in the presence of TβRII and TGFβ (ten Dijke *et al.* 1994a). This promiscuous association of ALKs with type II receptors is almost certainly an artefact of over-expression; however, the ligand-binding specificity for both activin and TGFβ is clearly conferred by the type II receptor. A chimeric type II receptor consisting of the extracellular domain of ActRII and the intracellular domain of TβRII specifically binds activin-A, and confers activin-binding on type I receptors (Ebner *et al.* 1993b). The signalling capacity of these various receptor complexes is addressed below.

Functional analysis of type I RSKs has led to the assignment of alternate names for various ALKs. ALK4 has been shown to mediate activin signalling and is often referred to as ActRIB (Attisano *et al.* 1996, Cárcamo *et al.* 1994, Willis *et al.* 1996). Initial observations suggested that ALK2 may also mediate activin signals and it acquired the label ActRI (Attisano *et al.* 1993). However, more careful analyses show that ALK2 is not an activin receptor, but instead may be a type I receptor for BMPs or MIS (Armes & Smith 1997, Cárcamo *et al.* 1994, He *et al.* 1993, Liu *et al.* 1995, Macías-Silva *et al.* 1998, ten Dijke *et al.* 1994b, Willis *et al.* 1996). ALK5 is the only demonstrated type I receptor for TGFβ and is therefore called TβRI (Franzén *et al.* 1993). ALK3 (BMPR-IA) and ALK6 (BMPR-IB) are BMP receptors (Koenig *et al.* 1994, Yamashita *et al.* 1995). ALK1 and ALK7 have not yet been assigned functional labels, although ALK1 may be a receptor for BMP7 (Macías-Silva *et al.* 1998). Based on sequence homology, these type I receptors group into three main classes; one includes ALKs 4, 5 and 7, a second is represented by ALKs 3 and 6, and a third is defined by ALKs 1 and 2.

10.2.3 RSK Activation

RSK types I and II form non-covalent, heteromeric complexes at the cell surface (Mathews & Vale 1993, Wrana *et al.* 1992). Analysis of a panel of mink lung epithelial cell (Mv1Lu) mutants lacking either functional type I or type II TβRs indicates that both receptor types are required for signalling (Wrana *et al.* 1992, Wrana *et al.* 1994b). Co-expression of ActRs with an activin/TGFβ-responsive reporter has indicated that both activin receptors are also required for this response (Cárcamo *et al.* 1994). Reciprocal co-immunoprecipitation of various ALKs and ActRII over-expressed in COS cells, and bound and cross-linked to [^{125}I] activin can be achieved using antibodies directed against either molecule (ten Dijke *et al.* 1994a). Interestingly, similar immunoprecipitation analysis of cells expressing native receptors revealed that affinity-labelled ActRII is co-immunoprecipitated only with antibodies against either ALK2 or ALK4 (ten Dijke *et al.* 1994a); TβRII co-immunoprecipitated only with ALK5. These observations indicate

that only a subset of the potential type I and type II receptor complexes actually form *in vivo*, implying a possible role for both ALK2 and ALK4 in mediating activin-specific responses, and suggesting that ALK5 is involved in TGFβ-specific signalling; as noted previously, however, ALK2 does not appear to signal activin responses (see above).

Whether heteromeric RSK complexes are pre-formed in the plasma membrane prior to ligand binding, or are formed from independent type I and type II receptors in response to ligand binding remains unclear. This issue cannot be addressed by the same affinity-labelling experiments used to define binding properties because this approach depends on the presence of radiolabelled ligand. Co-expression of ActRIIB and ALK4 in COS cells, followed by metabolic labelling with ^{35}S amino acids and immunoprecipitation has revealed that receptor I and II are associated independent of activin treatment. However, it is possible that ligand-independent receptor association occurs when the receptors are transiently over-expressed in cell culture (Attisano *et al.* 1996, Willis *et al.* 1996). Indeed, active complexes between ActRIIB and ALK4, and between TβRII and ALK5 can form in the absence of ligand when these molecules are co-expressed (Attisano *et al.* 1996, Chen & Weinberg 1995). However, it is clear that ligand promotes additional complex formation, phosphorylation of the type I receptors, and activation of the signalling pathway (Attisano *et al.* 1996, Lebrun & Vale 1997, Willis *et al.* 1996, Wrana *et al.* 1994a).

Because these receptors contain an intracellular serine kinase domain, much effort has been invested in determining the role of phosphorylation in receptor activation. The initial hypothesis was that both ActRII and type I activin receptors would be ligand-activated serine kinases. Biochemical characterisation of both type II ActRs and TβRII has indicated that these molecules are constitutively phosphorylated on serine residues, part of which is due to autophosphorylation (Attisano *et al.* 1996, Lin *et al.* 1992, Mathews & Vale 1993, Wrana *et al.* 1994a). ^{32}P-metabolic labelling of COS cells over-expressing ActRIIB alone, or in combination with type I molecules, yields equally phosphorylated receptor II with or without activin stimulation; tryptic phosphopeptide mapping patterns are identical in all situations. Several of these autophosphorylation sites in TβRII have been suggested to modulate the activity of the type II kinase, and Ser 213 appears to be required for

receptor activity (Luo & Lodish 1997). These observations indicate that type II receptor autophosphorylation activity is not regulated by ligand or receptor I, and that type II receptors are also phosphorylated by other intracellular serine/threonine kinases.

The phosphorylation state of type I receptors *in vivo* has been analysed following over-expression in mammalian cells. While ALK4 is phosphorylated to a low level when expressed alone, or in combination with receptor II, activin treatment leads to a rapid increase in ALK4 phosphorylation. Co-expression with an enzymatically inactive receptor II point mutant yields only basal levels of ALK4 phosphorylation, suggesting that type I receptors could be direct substrates for type II receptors (Attisano *et al.* 1996, Lebrun & Vale 1997, Willis *et al.* 1996). Phosphorylation of ALK5 by TβRII has also been measured following affinity purification of epitope-tagged receptor molecules (Wrana *et al.* 1994b). Ligand stimulation was reported to increase phosphorylation of receptor I; however, the phosphorylation state of ALK5 in the presence of TβRII and the absence of TGFβ have not been demonstrated. TβRII isolated by immunoprecipitation from both mammalian and insect cells was also shown to phosphorylate ALK5 *in vitro* (Wrana *et al.* 1994b, Ventura *et al.* 1994). These observations demonstrate that type I receptors are direct type II receptor kinase substrates.

The observation that type II receptors directly phosphorylate type I receptors led to the immediate search for the modified hydroxyamino acid residues within the type I receptors. The serines concentrated within the GS domain drew the most initial attention as predicted phosphorylation sites. Analysis of ALK5 indicated that mutation to valine or alanine of any two or more serines within the GS domain severely inhibited receptor signalling (Wieser *et al.* 1995). Similar analysis of ALK4 indicated that mutation of four of the five hydroxyamino acids in this region was required to inactivate the receptor, while mutation of S189A and S191A in combination was sufficient to significantly inhibit receptor function (Willis *et al.* 1996). These observations suggest that specific serines within this region may play a preferred role in receptor activation. Interestingly, it has also been shown that expression of increased levels of ActRIIB can rescue the signalling capacity of non-phosphorylatable ALK4 GS domain mutants; this suggests that phosphorylation of GS domain residues may not be absolutely required

for signal propagation, and that other phosphorylation sites may also play a role in ALK4 activation (Willis & Mathews 1997).

These analyses also demonstrated that mutation of Thr 204 in ALK5 to aspartic acid and mutation of Thr 206 in ALK4 to glutamic acid or aspartic acid resulted in constitutive activation of these receptors with regard to signalling (Attisano *et al.* 1996, Wieser *et al.* 1995, Willis *et al.* 1996). These constitutively active receptor mutants have proven quite useful in the ordering of RSK signalling events. For example, ALK5 (T204D) supports ligand-independent signalling in the absence of functional TβRII, and in the presence of dominant-negative enzymatically-inactive TβRII (Wieser *et al.* 1995). Similarly, ALK4 (T206E) mediates signalling in the absence of activin, or in the presence of a dominant-negative truncated form of ActRII (Attisano *et al.* 1996, Willis *et al.* 1996). These observations confirm that type I RSKs are downstream of type II RSKs.

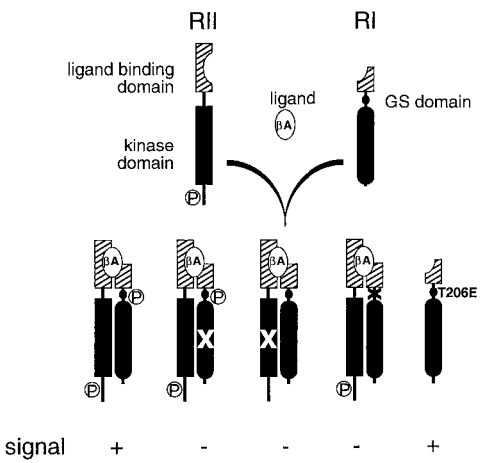

Fig. 10.2 Model for activin receptor activation by phosphorylation. Ligand stabilises receptor complexes, allowing ALK4 to be phosphorylated by ActRII at the GS domain and to transmit activin signals. A white "X" represents a point mutation which renders the receptor kinase domain enzymatically inactive. A black "X" at the GS domain represents a mutant receptor which cannot be phosphorylated at these residues. "T206E" denotes the point mutation within the GS domain which activates the type I receptor.

Collectively, these observations have led to the hypothesis that heteromeric receptor complexes are required to support signalling for both activin and TGFβ, with receptor II determining the ligand binding specificity of the complex. The following model for receptor complex activation has been proposed: type II receptors bind ligand at the cell surface, followed by recruitment of type I receptors into a heteromeric ternary complex, and subsequent phophorylation and activation of receptor I at sites within the GS domain (Fig. 10.2).

10.3 Activin Receptor Signaling

While the mechanism of receptor activation was being investigated, an intensive search was also being carried out to identify and clone molecular mediators of RSK signalling. Because the predicted substrate specificity of the RSKs differs from that of the well characterised receptor tyrosine kinases, the mediators of RSK signalling were expected to be novel. Adding to this interest in the identity of the RSK substrates was the large amount of data indicating that none of the classical signalling pathways or known second messengers seemed to be primarily involved in transducing TGFβ family signals (Table 10.1) (Mathews 1994). Genetic analysis in *Drosophila* and then in *C. elegans* yielded the first *bona fide* members of this signalling pathway, which are now known as Smads (Savage *et al.* 1996, Sekelsky *et al.* 1995). Another potential mediator was cloned in a yeast screen for novel activators of MAP kinase cascades (Yamaguchi *et al.* 1995); the physiological significance of this MAPKKK/Raf homologue (TAK1) and its activator, TAB1, in RSK signalling remains to be established (Shibuya *et al.* 1996). The yeast two-hybrid system was also employed in an effort to identify RSK interacting proteins; while a variety of molecules has been shown to interact with the receptor kinase domain in this system (Charng *et al.* 1998, Chen *et al.* 1995, Kawabata *et al.* 1995b, Oeda *et al.* 1998, Reddy *et al.* 1996, Wang *et al.* 1994), none of these currently appear to play a role in propagating RSK-initiated signals. Analysis of the Smad proteins has by far generated the most interest and the most convincing data in deciphering the RSK signalling pathways.

Table 10.1 Molecular mediators of TGFβ family signalling pathways. Known components of the respective ligand-initiated cascades are listed. This is not a comprehensive list, but rather an ordering of the most well characterised pathways.

Ligand	Type II RSK	Type I RSK	Pathway-Specific Smad	Common Smad
activin	ActRII ActRIIB	ALK4	Smad2 Smad3	Smad4
TGFβ	TβRII	ALK5	Smad2 Smad3	Smad4
BMP 2/4	BMPRII ActRII	ALK3 ALK6	Smad1 Smad5 Smad8	Smad4
BMP7	ActRII?	ALK1 ALK2	Smad1	Smad4
dpp	*punt*	*thick veins* *saxophone*	*Mad*	*Medea*
daf-7	*daf-4*	*daf-1*	*sma-2* *sma-3* *daf-8* *daf-14*	*sma-4*

10.3.1 Smad Proteins

Genetic analysis of the RSK signalling pathway in *Drosophila* and *C. elegans* led to the identification of the first true intracellular signal mediators for TGFβ-family ligands. The observation that different mutations in the *Drosophila* BMP homologue, *dpp*, result in phenotypes of graded severity was exploited in screens designed to isolate modifiers of weak *dpp* alleles. Two maternal effect enhancers of a weak *dpp* phenotype were isolated and named *Mothers against dpp* (*Mad*) and *Medea*, respectively (Raftery *et al.* 1995, Sekelsky *et al.* 1995). Molecular cloning and sequencing of *Mad* indicated that the gene coded for a protein with no previously described functional motifs, although the sequence was homologous to three predicted open reading frames in the *C. elegans* genome (Sekelsky *et al.* 1995). Subsequent cloning of the worm genes (*sma-2,-3 and -4*) based on shared mutant phenotypes of

small body size and male tail defects with RSK *daf-4* confirmed the presence of these homologous proteins (Savage *et al.* 1996). Although this protein family does not share homology with other known proteins, each member does contain highly conserved N- and C-terminal domains separated by a linker of variable length and sequence. The N-terminal domain has been called Mad-homology domain 1 (MH1), and the C-terminal domain has been designated Mad-homology domain 2 (MH2). Because these proteins lacked identifiable hydrophobic signal sequences or transmembrane domains, and because genetic mosaic analysis indicated that sma-2 is required autonomously in the same cells as daf-4, they were proposed to function as intracellular mediators of RSK signalling (Savage *et al.* 1996).

Following the genetic identification of these molecules, several vertebrate homologues were rapidly cloned using low-stringency hybridisation, PCR-based strategies, and EST database searches. One of the first human *Mad* homologues, *DPC4*/Smad4, was cloned independently in a search for genes homozygously deleted in pancreatic carcinoma; this observation led to the suggestion that these genes may function as tumour suppressors (Hahn *et al.* 1996). A murine homologue was cloned based on its functional capacity to change cell fate in *Xenopus* from ectoderm into mesoderm (Baker & Harland 1996). A unified nomenclature was soon adopted in which the original gene names from *C. elegans* (*Sma*) and *Drosophila* (*Mad*) were combined; the proteins are now known as Smads (Derynck *et al.* 1996).

Functional analysis of Smads in *Drosophila* and in *Xenopus* proved their role in mediating RSK signals downstream of receptors. The most compelling genetic evidence for placing Smads downstream of receptors is the capacity of *Mad* mutants to suppress the effects of dominant activating mutations in the type I RSK *thick veins* (Hoodless *et al.* 1996, Wiersdorff *et al.* 1996). Analysis of Smad function in *Xenopus* animal cap explants demonstrated that over-expression of xSmad1 resulted in ventralisation reminiscent of treatment with BMPs; conversely, over-expression of xSmad2 resulted in activin-like mesodermal dorsalisation (Baker & Harland 1996, Graff *et al.* 1996). Observation of similar effects of xSmad1 and xSmad2 in the presence of dominant-negative receptors provides additional evidence that Smads act downstream of receptors (Graff *et al.* 1996). These results not only demonstrated that Smads mediate TGFβ-family signals, but also provided

the first evidence that different Smads are responsible for transducing different ligand-specific responses.

Rapid progress in the biochemical analysis of Smad proteins followed their identification and biological characterisation. The first question to be addressed, based on the predicted enzymatic activity of the receptors, was whether Smads become phosphorylated in response to receptor activation. Smad 1 does become phosphorylated in cells treated with BMP2 or 4, but not with activin or TGFβ (Hoodless *et al.* 1996), Smads 2 and 3 are phosphorylated in response to activin or TGFβ, but not BMPs (Eppert *et al.* 1996, Macias-Silva *et al.* 1996, Zhang *et al.* 1996). The observation that Smad2 could be detected in immunoprecipitates with wild-type TβRII and enzymatically-inactive ALK5, but not with enzymatically-inactive TβRII and wild-type ALK5 suggested that Smads could interact directly with activated ALK5 and may be direct receptor substrates (Macias-Silva *et al.* 1996). Proof that type I receptors directly phosphorylate Smads was provided by showing that purified ALK3 could phosphorylate Smad1 and that purified ALK5 could phosphorylate Smad3 *in vitro* (Kretzschmar *et al.* 1997, Zhang *et al.* 1996). The site of inducible Smad phosphorylation was mapped to both Ser465 and Ser467 at the extreme C-terminus of Smad2 (Abdollah *et al.* 1997, Souchelnytskyi *et al.* 1997), and shown to be the same for *in vivo* and *in vitro* phosphorylation of Smad1 (Kretzschmar *et al.* 1997). Phosphorylation of these serines is required for normal Smad functions (discussed below), although the specific role that phosphorylation plays in mediating Smad activity remains unknown. Interestingly, Smad4 is not phosphorylated in response to ligand stimulation (Zhang *et al.* 1996); Smad4 also lacks the C-terminal serines characteristic of Smads 1, 2, 3, 5 and 8, which are designated pathway-specific Smads.

Phosphorylation of pathway-specific Smads is required *in vivo* for the manifestation of at least two normal Smad properties. The first of these is association with Smad4. Smad1 associates with Smad4 following activation of ALK3 (Kretzschmar *et al.* 1997, Lagna *et al.* 1996) and Smads 2 and 3 associate with Smad4 following activation of ALK4 or ALK5, respectively (Lagna *et al.* 1996, Nakao *et al.* 1997b); Smad phosphorylation site mutants do not associate with Smad4 *in vivo*. The requirement for Smad4 in signal propagation has been demonstrated using cell lines which lack functional

Smad4 protein; transcriptional responses to TGFβ and activin are observed only when exogenous Smad4 is provided (de Caestecker *et al.* 1997, Lagna *et al.* 1996, Zhou *et al.* 1998a). These observations are consistent with the genetic demonstration that multiple Smads are required in the same cells for normal tissue formation in *C. elegans* (Savage *et al.* 1996). *Medea*, the Smad4 homologue in *Drosophila*, has also been shown to be required for *dpp* signalling through *Mad* in flies (Das *et al.* 1998). Based on these functional criteria and comparative sequence analysis, Smad4 defines a second class of the Smad family which includes *Medea* and *sma-4*. Because Smad4 is required for the function of pathway-specific Smads, it is known as the "common" Smad.

Smads also accumulate inside the nucleus following RSK activation; Smad mutants which cannot be phosphorylated do not (Liu *et al.* 1997, Macias-Silva *et al.* 1996, Nakao *et al.* 1997b) function. Analyses using Smad4-deficient cells indicate that association with Smad4 is not required for the translocation of pathway-specific Smads (Liu *et al.* 1997). However, Smad4 does not accumulate in the nucleus in the absence of a phosphorylated partner, suggesting that Smad4 requires an activated pathway-specific Smad for nuclear entry (Das *et al.* 1998, Liu *et al.* 1997). Interestingly, isolated Smad2 MH2 domains are constitutively localised in the nucleus, and can induce mesoderm more potently than over-expressed full-length Smad2 (Baker & Harland 1996). This suggests that the MH1 domain or linker region either masks an unidentified nuclear localisation signal within MH2, or binds a protein in the cytoplasm which prevents nuclear accumulation. In any case, ligand-induced phosphorylation of pathway-specific Smads and subsequent association with Smad4 results in the nuclear accumulation of Smad heterooligomers.

A nuclear function for Smads was suggested by the demonstration that the MH2 domain of either Smad1 or Smad4 can activate transcription when tethered to DNA as a GAL4 DNA-binding domain fusion protein (Liu *et al.* 1996). Full-length Smad1 fused to GAL4 exhibited BMP-induced transcriptional activity, suggesting that pathway-specific Smads may function as ligand-dependent transcriptional regulators (Liu *et al.* 1996). Transactivation by Smad1 and Smad2 requires the presence of Smad4, indicating that Smad heterooligomers are also required for this function (Liu *et al.* 1997).

The hypothesis that Smads are physiological regulators of transcription was supported by the finding that Smad2 is a member of an activin-induced DNA-binding complex on the promoter of a *Xenopus* immediate-early response gene, *Mix.2* (Chen *et al.* 1996). The primary DNA binding component of this activin-dependent complex was found to be a novel winged-helix transcription factor designated FAST-1 (Forkhead Activin Signal Transducer) (Chen *et al.* 1996). Smad4 is also present in the complex and may serve to stabilise the direct interaction between Smad2 and FAST-1, which is mediated through the C-terminal domains of each protein (Chen *et al.* 1997a). A transcriptional reporter in which the activin-responsive element (ARE) from *Mix.2* drives expression of luciferase can be induced by TGFβ in mammalian cells, but only when FAST-1 is over-expressed (Hayashi *et al.* 1997). A human homologue of FAST-1 also supports induction of this reporter by TGFβ and activin when over-expressed, and the induction requires endogenous Smad4 (Zhou *et al.* 1998b). These observations confirm that Smads can function as transcriptional regulators within a ligand-induced sequence-specific DNA-binding complex.

Another independent activin-inducible DNA-binding complex forms on the promoter of the *Xenopus* early response gene *goosecoid.* This complex also contains Smad2 and Smad4, and an unidentified DNA-binding protein. The DNA sequence element bound by this complex is distinct from the ARE of *Mix.2* (Candia *et al.* 1997). A luciferase reporter under the control of *gsc* promoter elements is responsive to TGFβ and activin when a murine FAST-1 homologue, FAST2, is exogenously expressed. Surprisingly, over-expression of Smad3 blocks the FAST2-dependent induction of this reporter by TGFβ (Labbé *et al.* 1998). This represents the first reported difference in the function of ligand-stimulated Smads 2 and 3, and if substantiated, suggests complex regulation of TGFβ-dependent gene transcription.

Another observation that supports a role for Smads in transcriptional regulation is that the isolated N-terminal domain of *Drosophila Mad* can directly bind to specific DNA sequences in the quadrant enhancer of the *vestigial* gene. Furthermore, binding of *Mad* to this enhancer is essential for *dpp*-dependent transcription of *vg in vivo.* Interestingly, full-length *Mad* — expressed in and purified from bacterial cells — did not bind to DNA in these assays, suggesting that some modification is required to allow the full-length protein to recognise DNA (Kim *et al.* 1997).

Mammalian Smads 3 and 4 have also been shown to have sequence-specific DNA-binding activity, and optimal Smad binding elements (SBE) have been determined by oligonucleotide selection (Zawel *et al.* 1998). The consensus SBE (GTCTAGAC) is palindromic and can be found in the promoters of several activin/TGFβ-responsive genes with only minor differences (Dennler *et al.* 1998). Four tandemly repeated SBEs are sufficient to confer TGFβ-inducibility on a minimal promoter, which is wholly dependent on functional Smad4 (Zawel *et al.* 1998). In addition, mutation of the three SBEs in the human PAI-1 promoter abolishes TGFβ-responsiveness (Dennler *et al.* 1998). A Smad4 binding site is also found adjacent to the FAST binding site in the *gsc* promoter and is required for maximal transcriptional activation (Labbé *et al.* 1998). Interestingly, Smad2 does not appear to bind DNA like Smads 3 and 4 (Dennler *et al.* 1998, Labbé *et al.* 1998, Zawel *et al.* 1998); whether this represents a functional difference conferred by additional amino acids present near the DNA-binding motif in Smad2, or whether specific Smad2 DNA-binding sequences have simply not yet been identified remains to be determined. Taken together, these observations demonstrate that Smads can function as transcriptional regulators both by directly binding to DNA and by associating with other DNA-binding proteins.

Based on these observations, discrete functions have been ascribed to the conserved MH1 and MH2 domains (Fig. 10.3). In addition, the crystal

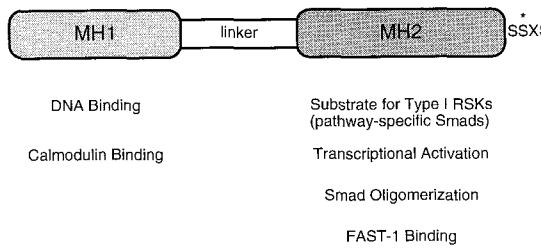

Fig. 10.3 Structural characteristics of the Smad proteins. Highly conserved *Mad* homology domains 1 and 2 are shown in different shades of grey. Stars mark the type I RSK phosphorylation sites within the pathway-specific Smad C-terminal sequence. Functional roles of each domain are listed below the schematic representation.

structures of isolated MH1 and MH2 domains have been determined, allowing functional descriptions to be interpreted in light of structural observations (Shi *et al.* 1997, Shi *et al.* 1998). The MH1 domain is clearly responsible for sequence-specific DNA-binding, with affinity toward the optimal SBE of between 1 and 5×10^{-7} M. Smads appear to bind DNA using a unique structural motif; a conserved 11-residue β hairpin provides base-specific DNA contacts within the major groove (Shi *et al.* 1998). The only other function of the MH1 domain described to date is association with the intracellular calcium sensor — calmodulin. Calmodulin appears to bind the Smad MH1 domain in a calcium-dependent manner, and inhibits Smad function as measured using a transcriptional reporter (Zimmerman *et al.* 1997); the physiological significance of this observation remains to be determined.

Point mutations which may interfere with normal Smad function have been identified in the MH1 domain of Smads 2 and 4. One such mutation in Smad4 (P130S) (Thiagalingam *et al.* 1996) lies within the hydrophobic core of the MH1 domain and probably results in substantial disruption of the overall folding pattern (Shi *et al.* 1998). Another mutation has been identified in both Smad2 (R133C) and Smad4 (R100T) (Eppert *et al.* 1996, Schutte *et al.* 1996); substitution of the corresponding residue in *Mad* has been reported to interfere with binding to the *vg* promoter (Kim *et al.* 1997), but does not alter binding of the Smad4 MH1 domain to the SBE (Shi *et al.* 1998). This residue is near to, but not within, the critical β hairpin, and has also been implicated in contributing to intramolecular interaction with the MH2 domain (Hata *et al.* 1997). Lying close to the previously mentioned residue in the three-dimensional structure, Smad4 (G65V) (MacGrogan *et al.* 1997) maps to a loop region of MH1 which may be important in mediating interaction with other proteins (Shi *et al.* 1998). It has been suggested that this region may interact with the MH2 domain and thereby limit MH2 domain transcriptional activity in the context of the unmodified full-length protein (Baker & Harland 1996, Hata *et al.* 1997); conversely, because the unmodified full-length protein binds DNA less well than the isolated MH1 domain, the MH2 domain has been described as inhibiting DNA-binding (Kim *et al.* 1997).

The MH2 domain of pathway-specific Smads serves as a type I RSK substrate (Kretzschmar *et al.* 1997, Macias-Silva *et al.* 1996), mediates Smad

homo- and heteromeric interactions and association with FAST proteins (Chen *et al.* 1997a, Lagna *et al.* 1996, Shi *et al.* 1997), and functions as a transcriptional trans-activator (Liu *et al.* 1996). The MH2 domain of Smad4 mediates interaction with Smads, and can activate transcription. The phosphorylated MH2 domain also mediates functional interaction with the transcriptional co-activators CBP and p300, which can augment Smad4-dependent transactivation (Feng *et al.* 1998, Janknecht *et al.* 1998, Pouponnot *et al.* 1998, Topper *et al.* 1998). The crystal structure of the MH2 domain suggests that Smads may function as homotrimers; three MH2 domains assemble into a trimeric disk with a loop, designated L3, protruding from one side and the MH1 domain presumably extending from the other (Shi *et al.* 1997). The L3 loop contains residues which determine specificity of receptor-Smad interactions (Lo *et al.* 1998), and mediates association between the pathway-specific and common Smads (Shi *et al.* 1997). The crystal structure also suggests that the large number of inactivating point mutations which cluster in the MH2 domain can be divided into three functional classes. The first class represents mutations in the L3 loop which interfere with Smad heterooligomer formation, a second class of mutations which map to the trimer interface prevents homooligomerimisation, and a third class includes residues required for the overall structural integrity of the molecule (Shi *et al.* 1997). Many of these mutations also prevent phosphorylation of the pathway-specific Smads, and all ultimately interfere with the formation of active heterohexamers.

In addition to the pathway-specific Smads and the common Smads, inhibitory Smads have also been identified. Smads 6 and 7, along with the *Drosophila* homologue, *Daughters against dpp* (*Dad*) function as negative feedback inhibitors of TGFβ family signalling; they are induced by the ligands which they inhibit, as well as by shear stress in vascular endothelium (Nakao *et al.* 1997a, Nakayama *et al.* 1998, Topper *et al.* 1997, Tsuneizumi *et al.* 1997). These Smads have much more divergent MH1 domains, but do share the conserved MH2 domain with the other Smad classes; inhibitory Smads lack the C-terminal phosphorylation sites of pathway-specific Smads.

Two mechanisms have been proposed for how these Smads exert their inhibitory function. Each of these Smads can bind to type I RSKs and block phosphorylation of pathway-specific Smads (Hayashi *et al.* 1997, Imamura

et al. 1997, Nakao *et al.* 1997a, Tsuneizumi *et al.* 1997). This prevents Smad heteromer formation and nuclear accumulation, and explains the non-specific inhibition observed when inhibitory Smads are over-expressed. A second mechanism accounting for specific inhibition of BMP signalling by Smad6 is based on the observation that Smad6 expressed at lower levels does not prevent phosphorylation of Smad1, but competes with Smad4 for binding to phosphorylated Smad1 (Hata *et al.* 1998). More analysis of endogenous inhibitory Smads will be required to refine these models. Another negatively acting Smad has been identified in *C. elegans* (*Daf-3*); however, based on the complexity of the genetic observations, *Daf-3* probably functions differently from the other inhibitory Smads (Patterson *et al.* 1997).

In summary, Smad proteins mediate RSK signals from the membrane into the nucleus (Fig. 10.4). Activation of RSKs occurs through ligand binding and complex formation between type II and type I RSKs. Type II

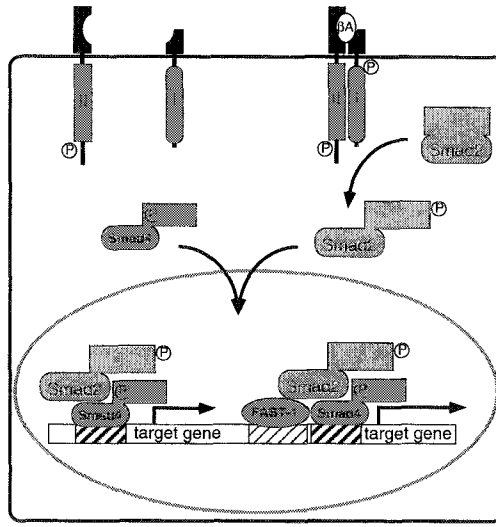

Fig. 10.4 Model for activin receptor signal transduction from the membrane to the nucleus. Ligand-bound phosphorylated ALK4 (I) phosphorylates the pathway-specific Smad2, which associates with the common Smad4 and moves into the nucleus. Activin-responsive target genes are induced either by direct binding of Smad complexes to the promoter, or by association of Smad complexes with other DNA-binding proteins such as FAST-1.

receptor-dependent phosphorylation of the type I receptor at the GS domain activates the type I receptor kinase which phosphorylates a pathway-specific Smad on C-terminal serines. The phosphorylated pathway-specific Smad associates with the common Smad4 in the cytoplasm and then moves into the nucleus where transcriptional activation occurs via direct DNA-binding by the Smad complex, or by association of the Smad complex with other DNA-binding proteins such as FAST-1. Inhibitory Smads negatively regulate this signalling pathway by binding to activated type I RSKs and inhibiting pathway-specific Smad phosphorylation, or by competing with Smad4 for binding to phosphorylated pathway-specific Smads and preventing formation of transcriptionally active Smad complexes.

10.3.2 Other Signal Mediators

While the Smads certainly mediate one primary pathway for RSK signal transduction, there is still great interest in the possibility that more than one effector is involved in generating the diverse functions of this ligand family. Many attempts at defining a potential role for MAP kinase family members in RSK signalling have yielded mixed results: TGFβ treatment has been reported to activate (Hartsough & Mulder 1995, Huwiler & Pfeilschifter 1994) to inhibit (Berrou *et al.* 1996, Howe *et al.* 1993), and to have no effect (Chatani *et al.* 1995) on the kinase activity of ERK; while activin appears to have no effect on ERK kinase activity (Graves *et al.* 1994, Sakurai *et al.* 1994). However, a genetic selection in yeast has been employed to identify a novel member of the MAPKKK family, which may be regulated by TGFβ and BMP4. TAK1 (TGFβ-activated kinase) kinase activity was induced three-fold in cells treated with TGFβ, and a kinase-inactive point mutant of TAK1 blocks TGFβ-dependent reporter gene activation (Yamaguchi *et al.* 1995). A TAK1-binding protein (TAB1) has also been cloned and shown to increase both TAK1 kinase activity and TGFβ-inducible reporter gene transcription in the absence of TGFβ (Shibuya *et al.* 1996). The relevance of theses proteins in physiological responses to TGFβ remains to be proven.

One of the first molecules to be cloned as a receptor kinase domain interacting protein using the yeast two-hybrid system was the immunophilin

FKBP12 (Wang *et al.* 1994). FKBP12 has been shown to interact with the cytoplasmic domain of most type I receptors both in yeast and in mammalian cells (Chen *et al.* 1997b, Okadome *et al.* 1996, Wang *et al.* 1994, Wang *et al.* 1996a); this interaction requires intact Leu193 and Pro 194 of ALK5 (Charng *et al.* 1996), and Gly 89 and Ile 90 of FKBP12 (Wang *et al.* 1996a). The initial hypothesis that FKBP12 would represent a type I RSK substrate and mediate signalling downstream of receptors now appears unlikely (Charng *et al.* 1996, Okadome *et al.* 1996, Shou *et al.* 1998). In fact, several observations indicate that FKBP12 serves as a negative regulator of type I receptor signalling activity. Increased type I receptor signalling capacity is manifested upon blocking the interaction between FKBP12 and ALKs by a variety of means including point mutations in either FKBP12 or type I receptors, or by inclusion of immunosuppressants FK506 or rapamycin (Charng *et al.* 1996, Chen *et al.* 1997b, Wang *et al.* 1996a). Whether ligand treatment results in FKBP12 release from the receptor complex is unresolved (Chen *et al.* 1997b, Okadome *et al.* 1996, Wang *et al.* 1996a), but FKBP12 does reduce phosphorylation of ALK5 when over-expressed with TβRII in cultured cells (Chen *et al.* 1997b). FKBP12 may regulate signalling by preventing phosphorylation/activation of ALKs in the absence of ligand, or by tethering other negative regulatory proteins to the RSK complex.

Several other proteins have also been identified as RSK-binding proteins using the two-hybrid system, but have still unproven roles in signal transduction. The only candidate which specifically interacts with a type II receptor is a WD domain protein called TRIP-1 (TGFβ receptor interacting protein 1) which interacts exclusively with TβRII (Chen *et al.* 1995). TRIP-1 is phosphorylated in the presence of over-expressed TβRII independent of ligand, and can function to selectively inhibit expression from TGFβ-inducible promoters (Chen *et al.* 1995, Choy & Derynck, 1998). The α-subunit of farnesyl transferase interacts with ALKs 2, 4 and 5 in yeast and in mammalian cells (Kawabata *et al.* 1995b, Ventura *et al.* 1996, Wang *et al.* 1996b); however, these ALKs do not interact with the farnesyl transferase holoenzyme, and farnesyl transferase activity is not altered in cells treated with TGFβ (Ventura *et al.* 1996). TRAP-1 (TβRI associated protein 1) associates specifically with activated ALK5 in yeast and human cells, and the

C-terminal ALK5-binding portion of TRAP-1 can suppress TGFβ-dependent gene expression (Charng *et al.* 1998). Finally, Apolipoprotein J has been shown to interact with both TβRII and ALKs 2 and 5 (Reddy *et al.* 1996), and two different Drosophila inhibitors of apoptosis have been shown to interact with the *Drosophila* type I receptor *thick veins* without demonstrating any biological function (Oeda *et al.* 1998). Whether these proteins are true RSK modulators, simple artefacts of the two-hybrid cloning system, or contain some common structural motif which is important for TGFβ signal transmission remains to be determined.

10.4 Inhibin Receptors

Activin is unique among the TGFβ family of ligands in that it shares one of two subunits with inhibin. Although activin acts on many more cell types than inhibin, in cells where both factors have a biological effect, their actions are antagonistic (Vale *et al.* 1990). This observation raises the fascinating question of how two factors which share an identical subunit can have opposing biological functions. Of course, detailed understanding of the molecular pathways that each factor employs would provide the most comprehensive answer to this question, but identification and cloning of inhibin signal mediators has proven to be quite challenging. The search for an inhibin receptor has been complicated by a variety of factors including the capacity of inhibin to bind to type II activin receptors, relative scarcity of bioactive inhibin, and the functional in-activation of inhibin by radiolabelling. In fact, it has been proposed that in-hibin antagonises activin function primarily by interfering with activin signal transduction. While such a mechanism does account for some inhibin effects, the observation that inhibin is up to ten-fold more potent than activin while binding to ActRIIs with ten-fold lower affinity suggests the presence of inhibin-specific mediators (Mathews & Vale 1991, Vale *et al.* 1986); evidence is mounting that specific cell surface binding proteins for inhibin do exist.

Inhibin binds to ActRIIs with affinity of about 1 nM, but does not bind to type I ActRs (Attisano *et al.* 1992, Lebrun & Vale 1997, Mathews & Vale 1991, Xu *et al.* 1995). Therefore, because of the requirement for RSK

complexes in signal initiation, binding of inhibin to ActRII and exclusion of ALK4 should result in dominant-negative inhibition of activin signalling. In fact, inhibin blocks activin-induced expression of a PAI-1 reporter gene and endogenous junB, haemoglobin synthesis in human erythroleukaemia cells, and inhibition of DNA synthesis in hepatic cells by competing with activin for binding to ActRIIs (Lebrun & Vale 1997, Martens *et al.* 1997, Xu *et al.* 1995). Furthermore, inhibition of activin-induced PAI-1 reporter expression was shown to be at the level of receptor function, and probably does not in-volve an inhibin-specific pathway; inhibin did not interfere with activin signals initiated by constitutively active ALK4 (Martens *et al.* 1997). These observations demonstrate that inhibin can act as a dominant-negative inhibitor of activin signalling, and provide a partial explanation of inhibin function. This function *in vivo*, of course, would be influenced by the local concen-trations of these factors in responsive tissues.

In addition to binding ActRIIs, inhibin also appears to bind specific cell surface proteins on a few responsive cell types. Inhibin binding sites have been identified on ovarian granulosa cells and testicular Leydig cells (Krummen *et al.* 1994, Woodruff *et al.* 1990). An inhibin-specific binding protein has also been observed in human erythroleukaemia cells which over-express ActRII and ActRIIB; while this protein is present in cells which do not over-express ActRIIs, it is much more readily detectable when increased amounts of ActRIIs are present (Lebrun & Vale 1997). The most compelling evidence for the existence of an inhibin receptor comes from analysis of murine stromal tumours resulting from targeted deletion of inhibin (Chapter 11). These tumours express higher levels of inhibin-specific binding proteins relative to ActRs and have been used as a tissue source for purification of a potential inhibin receptor. Four proteins from tumour homogenates were specifically retained on an inhibin affinity column, all of which bind inhibin, do not bind activin, and are not follistatin (Chapter 6) (Draper *et al.* 1998). Microsequence analysis of these purified proteins should allow for the cloning of the first inhibin receptors, and molecular characterisation of additional inhibin signal mediators.

10.5 Summary and Future Directions

The following model for activin signal transduction has emerged from the study of this pathway in a variety of model systems. Activin binds to and stabilises a heteromeric complex of transmembrane RSKs. Ligand-bound ActRII phosphorylates ALK4 (ActRIB) at residues within the GS domain, and at additional sites, which stimulates the enzymatic activity of ALK4. Cytoplasmic pathway-specific Smad proteins (Smad2 and/or Smad3) are then phosphorylated directly by ALK4 and form heterooligomers with the common Smad (Smad4) which accumulate inside the nucleus. Nuclear Smad heterooligomers regulate transcriptional responses by associating with DNA-binding proteins such as FAST-1, or by binding directly to Smad-specific DNA sequence elements. Inhibitory Smads can negatively regulate signalling by interfering with pathway-specific Smad phosphorylation, and by blocking formation of Smad heterooligomers.

Several interesting questions regarding activin signalling remain to be answered. Further investigation into Smad function will elucidate the specific role of phosphorylation in Smad activation, the basis for nuclear accumulation of Smad heterooligomers, and further define the structural motifs responsible for determining the specificity of Smad responsiveness. Identification of additional Smad regulatory proteins, and clarification of the role of calmodulin in inhibiting Smads will be pursued. There is also great interest in determining whether other pathways are regulated by RSKs. Do type II RSKs have any direct substrates, or do type I RSKs phosphorylate substrates other than Smads? Some of the molecules which might participate in RSK signalling have been mentioned and their roles will be investigated. The identification of an inhibin receptor remains one of the most interesting issues in the field. Will this receptor have a novel structure and signal via an unknown pathway, or will it represent an alternate partner for activin receptors? Progress to date has provided many of the tools required to address these questions and suggests that the answers will become known very soon.

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CHAPTER 11

TRANSGENIC MOUSE MODELS TO STUDY INHIBIN AND ACTIVIN

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11.1 Introduction

Inhibin, activin and follistatin were first identified as gonadal peptides regulating pituitary follicle stimulating hormone (FSH) secretion. Several studies have shown that these proteins have multiple functions in different cells and tissues. One of the methods of studying the precise role of a protein is the deletion of the gene. Inhibin and activin are composed of two subunits and the β-subunit is common for both proteins. This chapter reviews transgenic mouse models used to study the roles of inhibins, activins and follistatins giving insights into the importance of these proteins in non-reproductive functions.

11.2 Structure of Inhibin and Activin

The development of bioassays for the inhibins and the subsequent isolation of these compounds relied on the generation of radioimmunoassays (RIAs) for the gonadotropins. These RIAs permitted the identification of the tissues and subsets of cells that produced the inhibins. They also demonstrated that

these extracts worked specifically to regulate serum FSH but not luteinising hormone (LH). In 1985, several groups isolated and identified inhibin from both the bovine and porcine ovary (Ling *et al.* 1985, Miyamoto *et al.* 1985, Rivier *et al.* 1985, Robertson *et al.* 1985). The purified inhibin compounds were found to be heterodimers containing α and β subunits which were linked by several disulfide bonds. Protein sequence analysis allowed for the design of probes, leading to the isolation of cDNA and genomic clones for the two subunits (Mason *et al.* 1985, Mason *et al.* 1986b). The cloning of these subunit genes also led to the discovery of FSH-stimulating compounds, the activins, which were purified and determined to be homodimers of the β subunits (Ling *et al.* 1986, Vale *et al.* 1986). The inhibin α-subunit is transcribed from a single gene with several protein forms produced from various tissues (Robertson *et al.* 1985, Sugino *et al.* 1989). The β-subunit is the protein product of one of two different genes, classified as β_A and β_B, with both subunits approximately 14 kD in size (Ling *et al.* 1985, Miyamoto *et al.* 1985, Rivier *et al.* 1985, Robertson *et al.* 1985).

Comparison of the α- and β-subunit sequences with other known genes indicated that they shared a large degree of sequence homology with members of the TGF-β superfamily of signalling proteins. Besides the TGF-β genes, this family includes other important reproductive hormones such as growth differentiation factor 9 (GDF-9) (Dong *et al.* 1996), bone morphogenic proteins (BMP)-8a (Zhao *et al.* 1998), BMP-8b (Zhao *et al.* 1996), BMP-15 (Dube *et al.* 1998), and Mullerian inhibiting substance (MIS) (Behringer *et al.* 1994). Like the other members of this superfamily, inhibins and activins are dimers that are processed from pre-prohormones and have three or four intramolecular disulfide bonds. The dimerisation capabilities of the α and β subunits allow for the production of five different dimeric forms of this subfamily of proteins. Nomenclature for each individual compound is based upon which of the two β-subunit genes is employed in the hormone's production. For example, inhibin-A consists of an α-subunit heterodimerised to a β_A-subunit, while activin-AB consists of a β_A dimerising with a β_B-subunit (see Fig. 11.1). Both inhibins are believed to be equally bioactive, although their expression patterns are sexually dimorphic and change as the individual sexually matures, while the activins seem to have distinct and

unique roles in several embryonic and physiologic functions (Brown *et al.* 2000, Feijen *et al.* 1994, Tuuri *et al.* 1994, Woodruff *et al.* 1996).

The role of inhibins and activins in the hypothalamic-hypophyseal-gonadal axis has been well-defined (Vale *et al.* 1994). The hypothalamus releases gonadotrophin-releasing hormone (GnRH) onto the pituitary allowing for the release of LH and FSH from the gonadotrophs. FSH then binds to Sertoli or granulosa cells in the gonad and induces several gametogenic effects. Besides this gametotrophic effect, FSH also induces the production and release of inhibin. In several species, the adult female produces both inhibins A and B in response to FSH, while adult males reportedly produce only inhibin-B from the gonads (Andersson *et al.* 1997, Roberts *et al.* 1989, Shaha *et al.* 1989, Woodruff *et al.* 1996). The induced inhibin then binds to receptors in the pituitary and suppresses FSH production and secretion (Carroll *et al.* 1989, Corrigan *et al.* 1991, DePaolo *et al.* 1992). This positive-negative feedback loop is reversed when FSH is low. In the absence of FSH signalling, the gonad secretes activin, which can influence the gonad via paracrine and/or autocrine effects or upregulate FSH production from the pituitary (although this effect is probably not as physiologically relevant as activin produced by the pituitary) (Katayama *et al.* 1990, Rivier & Vale 1991). Upon release from the pituitary, FSH binds to gonadal FSH receptors. A cellular "switch" is then activated which signals the cells to upregulate the α-subunit (Feng *et al.* 1989, Pei *et al.* 1991). The α-subunit then dimerises with monomeric β subunits to produce inhibin at the expense of activin production. In this manner, the physiology of FSH production and its gametogenic effects are very tightly regulated.

The function of these hormones was originally believed to be limited to endocrine stimulation or repression of pituitary FSH. But, after their initial isolation, inhibin and activin were found to be produced in many other tissues and have a wider range of effects. Besides the gonad, the pituitary was also shown to produce and secrete inhibin and activin with autocrine effects in the gland subsequently described (Meunier *et al.* 1988, Roberts *et al.* 1989). Indeed, *in vitro* and *in vivo* data revealed these autocrine effects to be important in regulating basal levels of FSH production and secretion (Corrigan *et al.* 1991, DePaolo *et al.* 1992). Inhibin and activin also have

effects within the gonads themselves. Inhibin was initially thought to suppress meiotic maturation of oocytes, increase follicular recruitment, repress spermatogenesis, and regulate many aspects of steroidogenesis (Hsueh *et al.* 1987). Activin was determined to increase spermatogonial proliferation (Mather *et al.* 1990) and follicular atresia in the ovary (Woodruff *et al.* 1990, Yokota *et al.* 1997) while suppressing LH-induced steroidogenesis (Hsueh *et al.* 1987, Lin *et al.* 1989). The placenta, adrenal glands, bone marrow, and nervous system also express these proteins, leading to hypotheses about other possible actions outside the pituitary and gonad (Vale *et al.* 1994). Studies indicate that activin can stimulate GnRH, human chorionic gonadotropin (hCG), and progesterone release in placental cell cultures, while inhibin blocked these effects (Petraglia *et al.* 1989). In the brain, injections of activin into the hypothalamus stimulated a release of oxytocin into the bloodstream (Plotsky *et al.* 1991). Activin was also capable of *in vitro* stimulation of erythropoiesis (Yu *et al.* 1987) and has been shown to regulate liver metabolism and growth. *In vitro* studies have shown that activin-A can inhibit the synthesis of DNA and stimulate glycogenolysis in hepatocytes (Hully *et al.* 1994, Yasuda *et al.* 1993). *In vivo* experiments have shown that activin can induce hepatocellular necrosis about the central vein in both mouse and rat livers (Hully *et al.* 1994, Schwall *et al.* 1993). During embryogenesis, activin is present prior to and during gastrulation. Low levels of activin can also induce ventral mesoderm in *Xenopus laevis* (Green *et al.* 1992, Hemmati-Brivanlou & Melton 1992), although other members of the TGF-β family appear to be the true regulators of dorsal-ventral pattern formation in mammals.

The method by which these hormones transduce their signal has been elucidated in some detail (see Fig. 11.1). Inhibin and activin are produced as pre-prohormones, which are processed into their active dimeric forms in the endoplasmic reticulum and Golgi apparatus. Secretion from the cell allows the proteins to circulate freely or to form complexes with different binding proteins. Follistatin has been identified as one of these binding proteins and has been shown to play a major role in inhibin and activin signal transduction (Nakamura *et al.* 1990, Shimonaka *et al.* 1991). Indeed, follistatin attached to the target cell via heparan sulfate moieties, can bind

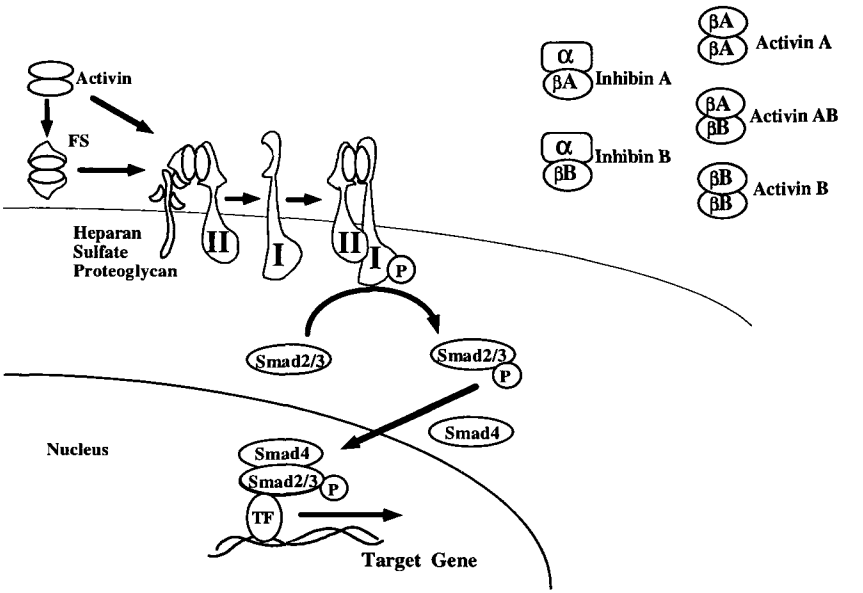

Fig. 11.1 Nomenclature and signal transduction of activin and inhibin. In the upper right-hand corner are the names and structures of the inhibins and activins. Inhibins are heterodimers of α and β subunits, while the activins are homodimers of β subunits. Follistatin (FS) binds the β subunits of activin (Nakamura *et al.* 1990, Shimonaka *et al.* 1991), with the complex found in the circulation or bound to heparan sulfate proteoglycans on the cell surface (Nakamura *et al.* 1990). Activin has been shown to bind two types of transmembrane receptors, classified as types I and II. Both of these receptor types are transmembrane serine-threonine kinases. Activin will bind to the type II receptor, which is unable to transduce a signal until subsequent recruitment of a type I receptor to the complex (Mathews 1994). This recruitment leads to the formation of a heteromeric complex with the receptors interacting via the activin moiety. The type II receptor then phosphorylates the type I receptor, which subsequently phosphorylates downstream signaling proteins including Smad2 and Smad3. These proteins then translocate to the nucleus with Smad4 and activate transcription of target genes (Chen *et al.* 1996, Chen *et al.* 1997, Mathews 1994, ten Dijke *et al.* 1994b).

activin and may subsequently "load" the ligand onto its receptors or act as a reservoir for activin (Nakamura *et al.* 1991). Activin has been shown to bind two types of transmembrane receptors, classified as types I and II based on their initial sizes on SDS-PAGE gels. Both of these receptor types are

transmembrane serine-threonine kinases. Activin will bind to the type II receptor, which is unable to transduce a signal until subsequent recruitment of a type I receptor to the complex (Mathews 1994). This recruitment leads to the formation of a heteromeric complex with the receptors interacting via the activin moiety. The type II receptor then phosphorylates the type I receptor, which subsequently phosphorylates downstream signalling proteins. Smad2 and Smad3 have been implicated as downstream proteins in the activin signal transduction pathway. Upon phosphorylation, Smad2 or Smad3 is activated and translocates with Smad4 to the nucleus to regulate activin responsive genes (Chen *et al.* 1996, Chen *et al.* 1997, Mathews 1994, ten Dijke *et al.* 1994b). There are two subtypes of activin type II receptors designated activin type IIA (ActRIIA) and activin type IIB (ActRIIB). In addition, two subtypes of type I receptors have been found to function *in vitro*, designated activin type IA (ActRIA or ALK2) and activin type IB (ActRIB or ALK4) receptors. In the adult, ActRIIA has been localised to many different tissues including the testes, ovaries, epididymis, vas deferens, uterus and pituitary (Cameron *et al.* 1994, de Winter *et al.* 1992, Feng *et al.* 1993, Mathews & Vale 1991). Expression in embryonic tissue has also been reported (Roberts *et al.* 1996). In contrast to activin, inhibin signalling pathways are less clear. Studies have shown that radiolabelled inhibin binding sites overlap some of the binding sites of activin (Krummen *et al.* 1994, Woodruff *et al.* 1992). This result may indicate that inhibin plays a dominant negative role over activin signalling (Lebrun & Vale 1997, Martens *et al.* 1997, Xu *et al.* 1995). However, inhibin-specific binding sites were also found using this method, indicating the presence of a putative inhibin receptor. The isolation of proteins possessing characteristics of putative inhibin receptors were recently reported (Draper *et al.* 1998, Chong *et al.* 2000, Lewis *et al.* 2000, Matzuk 2000). Further definitive characterisation is underway.

With the advent of embryonic stem cell technology and the ability to delete specific genes from the genome of mice, the studies of inhibin and activin physiology gained an additional dimension. This report is a summary of work in which genes involved in the inhibin and activin signalling cascades have been mutated in mice. Also reported are the results of crossing of these

Table 11.1

Mutation(s) [references]	Phenotype [references]
Activin Type IB Receptor (ActRIB)	Embryonic lethality [Gu *et al.* 1998]; development halted at the pre-gastrulation stage.
Activin Type IIA Receptor (ActRIIA)	Partial postnatal lethality [Matzuk *et al.* 1995c] with cranio-facial deformities; viable adult males exhibit delayed, fertility and females are infertile due to a block at the antral follicle stage.
Activin Type IIB Receptor (ActRIIB)	Postnatal lethality [Oh & Li 1997]; severe cardiovascular defect; loss of lateral asymmetry and axial skeletal defects.
Activin/inhibin β_A	Postnatal lethality [Matzuk *et al.* 1995d]; lack of whiskers, incisors, mandibular molars and palatal defects.
Activin/inhibin β_B [Vassalli *et al.* 1994] [Schrewe *et al.* 1994]	Hybrid background mice born with eyes open; females have an extended gestation period and were unable to nurse properly; increased serum FSH.
Activin/inhibin β_A/ Activin/inhibin β_B	Additive defects of β_A and β_B [Matzuk *et al.* 1995c] mutations.
Inhibin-α (Intact)	Gonadal tumours [Matzuk *et al.* 1992]; cancer cachexia-like syndrome [Matzuk *et al.* 1994] secondary to tumourigenesis [Matzuk *et al.* 1996]; majority die by 17 weeks of age.
Inhibin-α (Castrate)	Adrenal tumours after 21 weeks [Matzuk *et al.* 1992]; cancer cachexia-like syndrome [Matzuk *et al.* 1994] secondary to tumourgenesis [Matzuk *et al.* 1996]; median death at ≈33 weeks of age.
Inhibin-α/ActRIIA	Gonadal tumours after six weeks [Coerver *et al.* 1996] without cancer cachexia-like syndrome.
Inhibin-α/Mullerian inhibiting substance	Females similar to inhibin-α null females [Matzuk *et al.* 1995b]. Males had uteri, oviducts and proximal vagina (similar to MIS null mice), formed mixed sex cord-stromal tumours composed of granulosa/Sertoli cell and Leydig cell tumours.
Inhibin-α/Mullerian inhibiting substance receptor	Phenocopies the inhibin-α/Mullerian inhibiting substance Double null mutant mice [Mishina *et al.* 1996].
Inhibin-α/*hpg*	Similar to *hpg* mice; no tumour [Kumar *et al.* 1996] formation. Ovaries contain "Sertoli-cell only" tubules. Testes have a PAS-positive substance in seminiferous tubule lumen.
Inhibin-α/*tfm*	Females similar to inhibin-α null females [Shou *et al.* 1997]. Males similar to inhibin-α null males, although they showed increased longevity and less haemorrhagic tumours.

mutant mice into various genetic backgrounds in order to more fully understand the roles inhibin and activin play in reproduction, growth and embryogenesis. These studies are summarised in Table 11.1.

11.3 The Activin/Inhibin β_B Knockout Mice

The activin/inhibin β_B-subunit is a component of inhibin-B, activin-AB, and activin-B. Expression of the β_B monomer is found at multiple stages of embryonic and adult development and in several different tissue types (Albano *et al.* 1994, Green *et al.* 1992, Hemmati-Brivanlou & Melton, 1992, Vale *et al.* 1994, van den Eijnden-van Raaij *et al.* 1992). β_B mRNA and β_B immunoreactivity are present in the embryo during the pre-implantation stages of development (Roberts *et al.* 1996). In the adult, β_B is also produced in the gonads where its major sites of expression are Sertoli cells in males and granulosa cells in females. β_B-containing compounds are believed to have several intragonadal paracrine and autocrine effects along with extragonadal endocrine effects (Vale *et al.* 1994). Stimulation of spermatogonial proliferation and a role in spermatogenesis are known functions of activin-B within the testis, whereas in the ovary, activin has been shown to cause follicular atresia and stimulate granulosa cell proliferation. Extragonadally, activin-B is postulated to function in the inhibition of neural differentiation, the induction of erythropoetic differentiation, and the stimulation and inhibition of FSH production and secretion from the pituitary (Hashimoto *et al.* 1990, Hemmati-Brivanlou & Melton 1992, Murata *et al.* 1988, Schubert *et al.* 1990). Other known roles include the regulation of the secretion of oxytocin, adrenocorticotrophic hormone (ACTH) and growth hormone (GH) from the pituitary (Vale *et al.* 1994). To understand the functional role of the β_B-subunit in the physiology of the mouse, the gene was mutated utilising embryonic stem cell technology (Schrewe *et al.* 1994, Vassalli *et al.* 1994).

Heterozygote mutant mice displayed no overt phenotype, while homozygote β_B knockout mice exhibited developmental and reproductive deficits. Offspring showed a slight skewing of the expected 1:2:1 Mendelian

ratio, where homozygotes only represented 21% of the offspring in one genetic background, and an even lower percentage in other genetic backgrounds. The significance of this deviation is unclear.

The phenotypes of the β_B null mice range from defects in the prenatal fusion of the eyelids to major defects in reproductive physiology and fertility. An obvious phenotype of the β_B null mice was the birth of some mice with their eyes open. During normal development, the eyelids will fuse, and mice are born with their eyes closed. Penetrance of this phenomenon ranged from 0.00 to 0.84 across varied genetic backgrounds (i.e. the 129/Sv background did not demonstrate this defect, but most of the 129/Sv mice back-crossed into BALB/c or C57BL/6 backgrounds did). Mice born with their eyes unfused subsequently developed secondary eye defects that included hyperkeratinisation and squamous metaplasia of the corneal epithelium along with leukocytic infiltration of the cornea and eyelids. Permanent damage of the eyes in adult mice manifested in ocular dystrophy and opacification of the cornea. These results indicated that the loss of activin-B affects the prenatal development of the eyelid (inhibin α null mice were born with fused eyes, so this phenotype did not rely on the loss of inhibin-B).

Female β_B null mice also had reduced fertility, although there seemed to be no obvious ovarian defects. Indeed, the ovaries of β_B null mice showed no overt abnormalities and the pregnancy rate of β_B null mothers were similar to their heterozygote counterparts. The loss of fertility seemed to be the result of perinatal death of the offspring born to the β_B null mothers. When carefully examined, it was found that the offspring of these mothers rarely lived more than 24 hours, were cold and had empty stomachs. Nonviable offspring included wild type, heterozygote and β_B null mice, indicating that the defect was due to the mother's parenting. Although the β_B null mothers underwent lactation, they seemed to have problems with milk letdown (i.e. milk ejection). Offspring were rescued when placed with a foster mother confirming the problem as the mother's incapacity to nurse. Again genetic background influenced the severity of this phenomenon, but all strains of mice were affected. Another deviant aspect of the female's fertility was the gestation period. β_B null mothers experienced an average gestation period that was extended by one or two days. Some mothers never

initiated labour, became sick, and had their pups die *in utero*. Both parturition and milk letdown require oxytocin as an initiating factor, and activin has been shown to regulate oxytocin physiology. One possibility was that the defects in the secretion of oxytocin could be the cause of both of these phenomena. However, other studies (Robinson & Hennighausen 1997) and analysis of our oxytocin knockout mice (Nishimori *et al.* 1996) suggest that these defects are a direct local effect of absence of the activin β_B-subunit in the mammary gland and uterus. In contrast to the female β_B null mice, β_B null males did not exhibit any reproductive problems.

Male and female β_B null mice had altered endocrine profiles. In β_B null females, the ovary demonstrates a three- to 20-fold upregulation of the β_A-subunit. This effect was not accompanied by an increase in β_A mRNA, indicating that the effect was a post-translational increase in steady state β_A protein. Inhibin α protein levels were unaltered. Serum FSH concentration was also affected. FSH levels were increased by up to 20% in mutant males and females. This result may point to the loss of inhibin-B having a more dramatic effect on the pituitary (either locally or from the gonads) than the loss of both activins B and AB. These results are understandable when one considers that in the rat, inhibin-B is the only inhibin present in the adult male testes, and the predominant form of inhibin in adult female ovaries (Woodruff *et al.* 1996).

In summary, the activin/inhibin β_B gene is only required during embryogenesis for prenatal development of the eyelid. The loss of the β_B gene also affects the ability of females to undergo normal gestation and milk letdown. Finally, two endocrine pathways show altered homeostasis. Activin/inhibin β_A protein levels are increased in the β_B null ovary, while serum FSH is elevated in both males and females.

11.4 The Activin/Inhibin β_A Knockout Mice

The activin/inhibin β_A-subunit is a component of inhibin-A, activin-AB and activin-A. Similar to inhibin-β_B, expression of the β_A monomer is found at several different stages of mammalian development and in several different

tissues. β_A mRNA and immunoreactivity are similarly present in the pre-implantation stages of embryogenesis. After implantation, expression of β_A is primarily within the mesenchymal cells of the developing face, whisker and hair follicles, heart, and digestive tract (Albano *et al.* 1994, Feijen *et al.* 1994, Roberts & Barth 1994). In order to more fully understand the roles β_A plays in embryogenesis, embryonic stem cell technology was used to mutate the β_A gene in mice (Matzuk *et al.* 1995d).

Mice heterozygous for the mutant β_A gene were normal, viable and fertile, and β_A heterozygote crosses yielded the expected Mendelian ratio of 1:2:1 for β_A wild-type, heterozygote and homozygote null pups, indicating that the loss of the β_A gene did not result in embryonic lethality. However, β_A null mice had a phenotype that led to death within 24 hours of birth, despite the fact that they weighed and looked normal at birth. Consistent with the embryonic expression of β_A in the whisker follicles and teeth primordia, all of the β_A null mice lacked whiskers, incisors and mandibular molars accompanied by secondary defects in the alveolar ridge of the mandible. These mice also possessed various palatal malformations.

The lack of whiskers in β_A null mice was due to defects in the development of the whisker follicles. The whisker follicle arrays in these mice were normal. However, the development of the whiskers was abnormal as manifested in smaller hair follicles, hair papilla that reside closer to the skin surface, and the disruption of the cellular organisation about the base of the follicle. Interestingly, these whisker pads have their vibrissal follicles innervated, and the projection of these sensory afferents to the brainstem is topographically organised in a similar manner as compared to wild-type mice (Jhaveri *et al.* 1998). However, when the brainstem is histochemically stained for cytochrome oxidase, the β_A null mice do not exhibit any whisker-related patterns (barrelettes) in the trigeminal complex. This result indicates that loss of the activin/inhibin β_A-subunit leads to the follicle's atrophy, and the absence of vibrissal hair disrupts the activation of primary afferents and concommitant organisation of post-synaptic neurons in the brainstem trigeminal complex. Of course, an alternative explanation is that the brainstem is just delayed in setting up the proper organisational pattern of barrelettes, and the mice die before being capable of completing its formation.

β_A null mice also have aberrant tooth development. The incisors and mandibular molars had their development arrested in the bud stage, while maxillary molars developed normally. Interestingly, when activin A-soaked beads were implanted into the mandibles of β_A null mice, incisor and molar development were rescued (Ferguson *et al.* 1998). Tissue recombination experiments showed that the mandibular mesenchyme required activin prior to bud formation, while β_A null epithelia retained its ability to sustain tooth development as long as the mesenchyme delivered an activin signal. These results indicate that mesenchyme-derived activin-A is required to stimulate the mandibular epithelium and mesenchyme for proper mandibular tooth development. In support of this concept, activin type IIA and IIB receptors are present in both the mandibular epithelium and mesenchyme.

In addition to the aberrant whisker and tooth development, β_A null mice also demonstrated palatal defects (Matzuk *et al.* 1995d). Approximately 30% of β_A null mice had cleft secondary palates. Another 30% lacked a hard palate, with the remaining pups exhibiting a hard palate that was incomplete. Along with the whisker and tooth defects, these palatal defects led to the inability of β_A null pups to suckle, resulting in starvation and death.

The follistatin gene has also been mutated in mice (Matzuk *et al.* 1995e) yielding a homozygous phenotype with many similarities to the β_A null mice. Follistatin null mice had defective whiskers, abnormal incisors and cleft palates. These similarities indicate that proper craniofacial development is dependent on activin and follistatin interacting to establish the proper signalling events in mouse development. In addition to the defects shared with the β_A null mice, follistatin null mice have retarded growth, decreased mass of their diaphragm and intercostal muscles, shiny taut skin, and malformations of their ribs and vertebrae. These mice have difficulty breathing and die within hours of birth. These additional defects as compared to the β_A null mice indicate that follistatin may modulate the activity of other TGF-β family members. Our recent studies in which follistatin is over-expressed further supports this concept (Guo *et al.* 1998).

In summary, the β_A null mice died within 24 hours of birth as the result of multiple cranio-facial deformities, entirely consistent with the pattern of embryonic expression of the β_A gene during late embryogenesis. These

defects are shared by follistatin null mice. These similarities further establish the importance of activin-follistatin interactions in modulating activin signal transduction and development.

11.5 Activin/Inhibin β_A/β_B Compound Knockout Mice

β_A and β_B heterozygote mice were subsequently intercrossed to generate mice deficient in the activins A, B and AB and inhibins A and B (Matzuk *et al.* 1995c). These mice exhibited the additive effects of both β_A and β_B null phenotypes. Compound mutant mice were viable at birth, but died within 24 hours of birth. All lacked incisors, mandibular molars, and whiskers and were born with their eyes open. Palatal defects were present in all mice and were responsible for their subsequent death. No additional defects were found outside those expressed by the individual mutants separately. These results point to a lack of compensation of the two activin/inhibin β subunits for each other in the individual knockout phenotypes, and a lack of functional redundancy in activin physiology during embryogenesis.

11.6 Activin Type IIA Receptor Knockout Mice

As previously stated, activin is believed to transduce its signal through the binding of serine/threonine kinases termed type I and type II receptors. The current model of activin signal transduction involves activin binding to the type II receptor, which presents the activin to the type I receptor forming a heteromeric complex consisting of the receptors bridged by the activin dimer. The juxtaposition of these receptors then allows for the receptor-specific kinase activity to become activated inside the cell, transducing the signal across the cell membrane. Intuitively, one would then surmise that the loss of the activin ligand would not be the only way to block activin signal transduction. To test this hypothesis, the activin type IIA receptor (ActRIIA) gene was mutated using embryonic stem cell technology (Matzuk *et al.* 1995c). One hypothesis was that the ActRIIA null mice

would phenocopy the compound β_A/β_B null mice. The majority of ActRIIA null mice were viable at birth and overtly normal. However, at weaning, they comprised only 19% instead of 25% of the offspring of ActRIIA heterozygote intercrosses. When caesarean delivery was performed at E18.5, the embryos exhibited the proper Mendelian ratios, indicating the loss of some embryos was upon or after birth. In fact, 22% of the ActRIIA null embryos had a developmental defect, and all of these mice died within minutes of caesarean delivery. These malformations included cleft palate, defective eyelids, variable hypoplasia of the mandible (micrognathia), and defects in Meckel's cartilage. The latter two malformations are associated with aberrant development of the first branchial arch and were not present in the activin subunit null mice. These results suggest that an alternative ligand signals through ActRcIIA during mandibular development, although there is some compensation of another receptor (e.g. ActRIIB) in this process.

ActRIIA null mice which did not have developmental defects were overtly normal, but exhibited impaired reproductive capabilities. Male ActRIIA null mice were delayed in their onset of fertility, and female ActRIIA null mice were completely infertile. The ActRIIA null males exhibited reduced testis size and reduction in seminiferous tubule diameter and total volume. Interestingly, analysis revealed that spermatogenesis proceeded normally. The epididymes of 42-day-old mutant males were usually devoid of spermatozoa suggesting that the reduced seminiferous tubule volume was the likely cause of the delayed fertility. Female ActRIIA null mice exhibited thin uteri and small ovaries. Oocytes and follicular development were normal until the antral follicle stage when significant follicular atresia was noted. Consistent with this follicular block, there was a paucity of corpora lutea. These traits indicate that the ActRIIA null females rarely undergo normal oestrus cycles.

Profiles of pituitary hormones in the ActRIIA null mice showed a significant difference in the production and secretion of FSH as compared to wild-type controls, while LH remained unaffected. Both males and females were shown to have their serum and pituitary FSH levels decreased compared to wild-type controls. Meanwhile, LH was similar to wild-type controls.

The reduction in serum FSH in the mutant females probably accounts for the aberrant oestrous cycles and concomitant infertility. In the mutant males, spermatogenesis occurs despite the low serum FSH concentration and the lack of signalling through ActRIIA. Lastly, the findings in both males and females are consistent with the defects seen in FSH null mice (Kumar *et al.* 1997) and suggest that absence of ActRIIA acts like a hypothetical FSH hypomorph mutation.

In summary, the ActRIIA null mice do not phenocopy the β_A/β_B compound null mice. Most of the activin type IIA receptor null mice undergo normal embryogenesis. Subsequently, they develop reproductive defects that in the male delays the onset of fertility, while in the female completely blocks it. The likely cause for the reproductive defects is the loss of signalling through ActRIIA in the pituitary. This absence of signalling through the ActRIIA reduces the synthesis and secretion of FSH, which is required for normal fertility. Some of the ActRIIA knockout mice show unique craniofacial malformations. The likelihood is that other receptor complexes are compensating for the loss of the ActRIIA. The unique defects (i.e. mandibular defects) point to the formation of ActRIIA complexes with other ligands during the crucial period of development of the first branchial arch.

11.7 Activin Type IIB Receptor Knockout Mice

The activin type IIB receptor (ActRIIB) functions in a similar manner as the activin type IIA receptor. Upon ligand binding, ActRIIB forms a heteromeric complex with activin type I receptors with subsequent intracellular signal transduction. The two receptors are 50–60% identical in the ligand-binding domain while sharing 60–70% identity in their kinase domains (Attisano *et al.* 1992). Their expression patterns are unique, but demonstate some temporal and spatial overlap. For example, ActRIIA is expressed only on the right side of Henson's node during chick development, while ActRIIB expression is found on both sides of this structure (Levin *et al.* 1995). ActRIIB is also expressed in the epiblast of pre-gastrulation mouse embryos, the embryonic ectoderm, and during organogenesis in several tissues including

brain, spinal cord, limb, metanephros, stomach and intestine (Feijen *et al.* 1994, Manova *et al.* 1995). Numerous experiments have elucidated the role of ActRIIB during embryogenesis. Over-expression of a truncated ActRIIB (tXAR) blocked activin-mediated mesoderm formation, while injection of this truncated receptor into the left-dorsal blastomere of 16-cell-stage *Xenopus* embryos randomised heart situs (Hyatt *et al.* 1996). These results suggested that ActRIIB may be important in left-right axis patterning and development, even though activin ligand is not required for these events (Matzuk *et al.* 1995c). This idea further supports additional ligands in signalling through ActRIIB. In order to more fully understand the role ActRIIB plays in these events, the ActRIIB gene was mutated using ES cell technology (Oh & Li 1997).

Loss of the ActRIIB gene results in postnatal lethality due to defects in cardiovascular and axial skeleton formation. Most animals were born cyanotic and died within hours of birth. Cyanosis is indicative of cardiovascular defects, and upon autopsy many malformations of the heart and vascular system were discovered in the ActRIIB mutants. ActRIIB null mice had ventricular and septal defects. In addition, the two great arteries, the aorta and pulmonary trunk, were in abnormal positions similar to human congenital heart conditions identified as transposition of the great arteries (TGA) or double outlet right ventricle (DORV). In the 129/Sv inbred mouse background, 100% of the ActRIIB null mice had these defects, but in a hybrid background (129/Sv-C57BL/6), the defects were seen only 60% of the time. The heart was also found to have a randomised positioning with regard to the direction of the apex. Independent of the position of the apex, the direction of aortic looping was randomised in these mice. The frequency of this randomisation of direction and positioning was also greater in the 129/Sv background. Besides these cardiovascular malformations, the ActRIIB null mice expressed a number of other congenital defects. These mice had smaller and abnormally shaped spleens, altered liver lobation, right pulmonary isomerism, and bilateral right atrial appendages consistent with the human asplenia syndrome (or right isomerism). The left lung contained the same number of lobes as the right lung, which is characteristic of right pulmonary isomerism. The kidneys showed unilateral or bilateral agenesis, along with hypoplasia. Viable mice

(i.e. mice that lived past the first day of birth) did not exhibit these defects. These results indicate that ActRIIB plays a major role in the initiation of left-right asymmetry and organogenesis.

The axial skeletons of the ActRIIB null mice were also malformed. These malformations were found in both viable and non-viable ActRIIB null mice. These mice exhibited an increased number of thoracic vertebrae associated with increased numbers of vertebral-sternal and free ribs. When Hox gene expression was examined, ActRIIB null mice were shown to have similar levels of expression of Hox- 6, 8, 9 and 10, although the spatial patterning of expression was skewed in a posterior direction. Low doses of all-trans retinoic acid (RA) during pregnancy have also been shown to induce the anterior transformation of the thoracic vertebrae (Kessel & Gruss 1991). Exposure of mice to RA during pregnancy has also been shown to induce cardiac malformations (TGA or DORV), pulmonary isomerism and altered Hox gene expression pattern in mice (Kim *et al.* 1995, Yasui *et al.* 1995). When ActRIIB null mice were exposed to RA *in utero*, they had even greater increases in the number of thoracic vertebrae (Oh & Li 1997). Interestingly, upon RA treatment, the ActRIIB mice lost some caudal and even sacral vertebrae, while expressing an increased incidence of spina bifida. Wild-type mice exposed to RA did not show these defects unless the RA concentration was increased five- to ten-fold. These results indicate that the loss of the ActRIIB also leads to anterior transformation of the vertebrae, which when combined with RA has synergistic effects on the patterning of the axial skeleton.

In summary, Oh & Li (1997) show that ActRIIB is a vital regulator of murine development. Gross malformations of several systems resulted in embryonic lethality. Additionally, the loss of ActRIIB allows for the alteration of axial patterning in these mice. These results are in contrast to the data generated from mice lacking either activin ligand (Matzuk *et al.* 1995c). These phenotypic differences suggest that other ligands act via the ActRIIB during mouse embryogenesis. In *Xenopus laevis*, one potential factor is Vg-1. The tXAR receptor has also been shown to block Vg-1 mediated mesodermal formation (Schulte-Merker *et al.* 1994). Vg-1 injected into the right-dorsal blastomere of 16-cell stage *Xenopus* embryos caused randomised

heart situs in manner similar to when ActRIIB was injected into the left-dorsal blastmere (Hyatt *et al.* 1996). In mammals, nodal and nodal-related peptides are more likely the relevant ligands which signal through the ActRIIB during these early stages.

11.8 Activin Type IB Receptor (ALK4) Knockout Mice

Type I activin receptors require the presence of type II activin receptors to initiate signal transduction in the presence of activin. Activin type IA receptor (ActRIA, also known as ALK2, R1 and Tsk 7L) binds activin in conjunction with the ActRIIs, while also being capable of binding BMPs in the presence of BMP type II receptor (Liu *et al.* 1995, ten Dijke *et al.* 1994b). The activin type IB receptor (ActRIB, also known as ALK4 and R2) is believed to be functional only in the context of ligand-bound activin type II receptors (Attisano *et al.* 1993, ten Dijke *et al.* 1993, ten Dijke *et al.* 1994a). Studies have shown that a kinase-deficient ActRIB is unable to stimulate activin-induced transcriptional activity (Tsuchida *et al.* 1993). In addition, over-expression of ActRIB in developing *Xenopus laevis* initiates dorsal mesoderm, while a kinase-deficient ActRIB inhibits activin-induced mesoderm formation (Armes & Smith 1997, Chang *et al.* 1997). In order to elaborate and more fully understand the role of ActRIB in mouse development, targeted disruption of the ActRIB gene was performed using ES cell technology (Gu *et al.* 1998).

The loss of a functional ActRIB gene in mice results in embryonic lethality that appears to be caused by a failure to properly undergo gastrulation. During mouse development, ActRIB is expressed in the epiblast and the extraembryonic ectoderm (Gu *et al.* 1998). The epiblast is the site of primitive streak formation, with mesoderm formation being the result of the migration of epiblast cells through the primitive streak. The loss of ActRIB lowers the number of epiblast cells in the embryo and also dissociates the epiblast cells from the visceral endoderm layer. In addition to these defects, the epiblast and extraembryonic ectoderm are ectopically located, the extraembryonic ectoderm does not form a cavity, and there is a lack of squamous visceral

endoderm. These defects lead to a disruption and malformation of the egg cylinder prior to gastrulation resulting in embryonic lethality. A similar phenotype is seen in mice that have had the nodal gene deleted (Conlon *et al.* 1991, Zhou *et al.* 1993).

To better understand the effects that ActRIB has on gastrulation and development, chimeric embryo analysis was performed. In these studies, ActRIB null ES cells were injected into wild-type blastocysts, while wild-type ES cells were also injected into ActRIB null blastocysts. The injected ES cells would be expected to contribute to the formation of epiblast, which will develop into the embryo proper, and extraembryonic mesoderm, but not the trophectoderm and primitive endoderm. When ActRIB null ES cells were injected and subsequently grew to comprise 10–50% of the chimeric embryo, most of the embryos were found to be normal from stages E6.0–E9.5, although the ActRIB null cells were found in small clusters that were located primarily in the posterior and lateral mesoderm. In addition, analysis at E7.0 found that ActRIB null cells did not contribute to early mesodermal formation. This result shows that ActRIB is not required for a cell to become mesodermal, although it is required for formation of early mesoderm. When ActRIB null cells made up over 80% of the epiblast, primitive streak formation failed and embryonic mesoderm was not formed. This defect could be rescued by the presence of a transgenic human ALK4 gene. When wild-type ES cells were injected into ActRIB null blastocysts, no embryonic tissues were formed. In contrast, the extraembryonic tissues were mostly normal and the extraembryonic mesoderm was produced. These results indicate that gastrulation is severely disrupted. If ActRIB was required only for formation of the epiblast, the large wild-type cell contribution to the embryo should have allowed for proper gastrulation. Because the wild-type cells did not rescue the gastrulation defect, ActRIB then must also function in the extraembryonic structures during gastrulation and send a signal to the embryo. The results indicate that the expression of ActRIB in the epiblast and the extraembryonic tissues has a significant role in the formation of the primitive streak during gastrulation.

Of some interest is that the activin ligand null mice undergo proper gastrulation and are born alive (Matzuk *et al.* 1995c). If ActRIB is specific

for activin, how could the compound activin/inhibin β_A and β_B subunits null mice develop to term? One explanation is that ActRIB is not specific for the activin signal cascade. The similarities between the ActRIB null and nodal null mice suggest that ActRIB is a component of nodal signal transduction. Recent studies with ActRIIA/nodal double mutant mice suggest that at least ActRIIA is a component of the nodal signaling cascade (Song *et al.* 1999).

11.9 Inhibin-α Knockout Mice

The inhibin α-subunit is a component of inhibins A and B. Inhibins are proteins that are antagonists of activins in many tissues. Intragonadally, inhibin has paracrine effects that decrease spermatogenesis, suppress meiotic maturation of oocytes, increase follicle recruitment, and modify steriodogenesis (Vale *et al.* 1994). Extragonadally, inhibin represses the production and secretion of FSH from the pituitary, block trophoblast synthesis of hCG and progesterone, and inhibit erythropoesis *in vitro* (Vale *et al.* 1994). Furthermore, inhibin-α mRNA and protein are found in bone marrow, adrenal glands and the nervous system. To further dissect the roles of inhibin in developmental and reproductive physiology, the inhibin α-subunit was mutated using ES cell technology (Matzuk *et al.* 1992).

Inhibin-α null mice display a rather striking phenotype; they are normal at birth but within weeks develop gonadal sex-cord tumors with 100% penetrance. The initiation of tumorigenesis is accompanied by a wasting syndrome, which is similar to human cancer cachexia-like syndromes (Matzuk *et al.* 1994). Castration temporarily rescues the individuals from this gonadally-induced wasting syndrome, but the mice subsequently develop a new cachexia-like syndrome and perish. Autopsies revealed that the return of the wasting syndrome in the castrate mice is caused by the formation of adrenal cortical tumours (Matzuk *et al.* 1994). The results indicate a role for inhibin-α as a putative tumour suppressor gene for the gonads and the adrenal glands.

Inhibin-α null mice display no abnormalities at birth, undergo normal sexual differentiation, and exhibit normal external genitalia. Heterozygote

crosses yield litters of normal size consisting of mice representing the proper Mendelian frequencies. As early as four weeks of age, male and female inhibin-α null mice begin to form gonadal tumours (Matzuk *et al.* 1992). These tumours consist of cells displaying a mixed and incompletely differentiated gonadal sex cord-stromal (granulosa/Sertoli cell) phenotype. Macroscopically, the gonads enlarge with the tumour load and display multiple haemorrhagic foci. Microscopically, the testes show intratubular lesions that are often haemorrhagic and resemble human juvenile granulosa cell tumours. Spermatogenesis is active in younger male inhibin knockout mice, but regresses in proportion to the increase in tumour mass. This blockage is bilateral, even when the tumour is only unilateral, indicating the presence of a soluble factor affecting this function. In addition, a reduction in the number of Leydig cells is evident at this age. In the female, the ovaries have tumours that are mixed nodular masses displaying undifferentiated "granulosa cell" derivatives. They also contained mitotically active cells in a seminiferous tubule-like arrangement. Besides these lesions, some ovaries also contain large haemorrhagic cysts lined with a flat, nondescript epithelium. No tumourous foci are associated with these cysts. Metastasis was never noted for the gonadal tumours.

Soon after the development of the gonadal and adrenal tumours, the inhibin-α null mice develop a cancer cachexia-like syndrome and die (Matzuk *et al.* 1994). Severe weight loss, lethargy, a pale periphery, and thoracic kyphoscoliosis are the grossly obvious external features of the syndrome. Internally, the liver undergoes a non-tumourous degeneration in mass and size and exhibits a pale brown colour. Microscopic investigation reveals the liver to be uniformly micronodular with enlarged hepatocytes containing swollen nuclei. Hepatocellular necrosis is evident about the central vein and is associated with foci of chronic lymphocytic infiltration. Blood samples showed increased levels of hepatocellular enzymes and a decreased hematocrit. Hematocrits revealed the mice contained normal levels of red blood cells at four to six weeks of age, but at the onset of tumourigenesis was lowered from 44% to 28%. There was also a reduction in the number of leukocytes and platelets (pancytopenia). The glandular stomach also displayed non-tumourous microscopic changes consisting of an atrophied

mucosal layer and a marked depletion of parietal cells. The stomach pathology was caused by a block in the differentiation of neck cells to pepsinogen-secreting zymogen cells, and the terminal differentiation of pre-parietal cells to acid-secreting parietal cells. There is also a significant increase in the number of pre-pit and pit cells per gastric unit, along with an increase in the number of pre-caveolated and caveolated cells. Although terminal differentiation of the pre-caveolated cells is complete, the pit cell lineage does not undergo complete maturation (Li *et al.* 1998). The stomach pathology could be an important factor in the development of the wasting syndrome. With the loss of parietal cells, the acidity of the stomach is diminished, lowering the efficiency of iron absorption, and perhaps inducing or worsening the anaemia in these animals. Parietal cells also secrete intrinsic factor into the lumen of the stomach. Loss of intrinsic factor would compromise the intake of vitamin B12, furthering the illness in these mice. This syndrome eventually weakens the health of the mice and leads to death by 12 weeks of age in 95% of the males and 17 weeks of age in 95% of the females.

Castration of the inhibin-α null mice stopped the progression of the cancer cachexia-like syndrome with a subsequent return to health of the affected mice (Matzuk *et al.* 1994). However, weeks later, a re-development of the wasting syndrome occurred, and the mice again exhibited signs of cachexia and died. Autopsies revealed that these mice had developed tumours of the adrenal cortex, with the earliest of the adrenal tumours found at 21 weeks of age. The tumours were primarily unilateral, haemorrhagic, and composed of a mixed population of undifferentiated and steroidogenic cells. In contrast to the gonadal tumours, metastasis to the lung and liver was present in a few cases. The cancer cachexia-like syndrome was similar to the one accompanying the gonadal tumours with identical macrosopic and microscopic findings.

Altered endocrine profiles were also factors in the health and survival of the inhibin-α null mice. The tumours in these mice were shown to secrete large amounts of activin into the bloodstream. Indeed, when compared to wild-type controls, serum activin-A levels were 13- and 20-fold higher in inhibin-α null males and females, respectively. Serum activin-B levels were also dramatically elevated above normal. These increases in activin levels

are the cause of the liver degeneration and stomach pathology (see Sec. 11.10). Previous studies demonstrated that injections of activin-A induced the same alterations of liver function that is found in mice undergoing the cachexia-like syndrome (Hully *et al.* 1994, Schwall *et al.* 1993, Yasuda *et al.* 1993). FSH levels were also elevated two- to three-fold in inhibin-α null mice. This rise in serum FSH is probably another downstream effect of the unopposed local intrapituitary increase in activin-B and the high serum activin levels. Finally, estrogen levels were also elevated in nearly all of the inhibin-α null male and female mice (Matzuk *et al.* 1996). This phenomena is due to the production of aromatase in the "granulosa cell" component of the tumours converting androgens to estrogens and resulting in increased serum concentration of these steroids. The bilateral block in spermatogenesis that occurs with the increase in tumour size may be the result of these elevated estrogen or activin levels or another soluble factor that has yet to be identified. Any additional effects that these elevated endocrine levels could have on the physiology of the inhibin-α null mice are discussed in greater detail later in this chapter.

The fertility of the inhibin-α null mice was also investigated (Matzuk *et al.* 1996). Some inhibin-α null males show a delay in the progression of the cachexia-like syndrome. These individuals are believed to have a later onset of tumourigenesis. Prior to tumourigenesis, the prostate, seminal vesicles, testes and epididymes of mutant males have normal architecture. These mice also exhibit a slight decrease in Leydig cell number. Spermatozoa are present in the lumen of the seminiferous tubules and epididymes. When mated to superovulated females, four out of four inhibin-α null males produced plugs which led to the subsequent fertilisation of the eggs. However, upon tumuor formation, the seminiferous tubules degenerate and undergo spermatogenic failure. As previously stated, spermatogenic failure occurs bilaterally even when the tumour is unilateral. In addition, tumour formation causes a degeneration of the seminal vesicles.

Fertility in inhibin-α null females was also investigated (Matzuk *et al.* 1996). In three- to four-week old inhibin-α null females, superovulation studies revealed that ovulation can occur and that the eggs were capable of being fertilised. However, all of these capabilities were dramatically decreased

in comparison to wild-type and heterozygous controls. Females tested at six weeks of age were infertile (all had developed tumours). Histological analysis revealed that inhibin-α null ovaries contained some antral follicles, but did not contain corpora lutea. Older females often had fluid filled uteri that was probably a secondary effect of the elevated estrogen levels. These experiments indicated that the inhibin-α null male mice, but not the female mice, had the capacity to reproduce for a period of time prior to the formation of tumours.

Transplantation studies were carried out to determine if the mutant ovaries could be rescued from tumourigenesis (Matzuk *et al.* 1996). These studies would also determine if the lack of inhibins or the elevated activins were responsible for inducing tumourigenesis. Ovaries from three-week-old female inhibin-α null mice were removed and transplanted into immunocompatible wild-type females that were either unilaterally or bilaterally ovariectomised. Ovaries transplanted into bilaterally ovariectomised females were never exposed to any inhibins, and subsequently exhibited tumours and the wasting syndrome in four out of four cases. Ovaries transplantated into unilaterally ovariectomised females (i.e. the mutant ovaries were exposed to circulating inhibins) did not form tumours in ten out of ten cases up to a period of 14–33 weeks after transplantation. However, five out of ten of these mice formed "Sertoli tubule" structures in the ovary. No mature Graafian follicles or corpora lutea were present, indicating that no "end stage" follicular development occurred. The results of these studies indicated that the absence of the inhibins, and not the presence of elevated activin production, was responsible for the generation of gonadal tumours in the inhibin-α null mice. The breeding of inhibin-A inducible transgenic mice to the inhibin-α null mice has also confirmed that over-production of inhibin-A can suppress testicular tumourigenesis (Pierson *et al.* 2000).

The deletion of the inhibin-α gene in mice leads to a phenotype that provides insight into the roles of inhibin in reproductive physiology and tumour suppression. The formation of gonadal tumours in all mutant mice indicates that the inhibins are essential proteins for regulating the growth of specific gonadal and adrenal cells. The loss of the inhibin α-subunit allows for the activin/inhibin β subunits to homodimerise and form activin. This latter event allows for the increase in serum activin to extraphysiologic

levels. In experiments discussed further in the next section, these elevated activin levels act like a "gain of function" mutation that is responsible for the onset of the cancer cachexia-like syndrome that eventually kills the mutant mice (Coerver *et al.* 1996). These elevated activin levels also contribute to the increased levels of FSH, which along with the high activin levels, likely increase the speed and aggressiveness of these tumours. Thus, the inhibin-α null mice have provided useful information in the study of reproductive endocrinology and tumourigenesis.

11.10 Inhibin-α/Activin Type IIA Receptor Knockout Mice

The hepatocellular changes that accompany the cancer cachexia-like syndrome in the inhibin-α null mice are reminiscent of changes that occur when recombinant activin-A is injected into mice and rats. Activin has been found to affect the liver in a number of ways. *In vitro* studies have shown that activin increases glycogenolysis in hepatocytes, while *in vitro* and *in vivo* studies have shown that activin can block initiation of DNA synthesis and induce apoptosis in hepatocytes (Hully *et al.* 1994, Mine *et al.* 1989, Schwall *et al.* 1993, Yasuda *et al.* 1993). Activin type IIA receptor mRNA has been detected in the liver, and the liver is a major site of ^{125}I activin binding (Mathews & Vale 1991, Matzuk *et al.* 1995d, Woodruff *et al.* 1993). With this information, it was postulated that the increased serum levels of activin in the inhibin-α null mice were responsible for the wasting syndrome present in these mice. To determine if this hypothesis was correct, double mutant mice lacking both inhibin-α and the activin type IIA receptor genes were created (Coerver *et al.* 1996). These double mutant mice exhibited gonadal tumours but did not exhibit any signs of the cancer cachexia-like syndrome. Life-spans were increased beyond those seen with the mice lacking only the inhibin-α gene, indicating that in the absence of signalling through activin type IIA receptor, there was no wasting syndrome.

The majority of the inhibin-α/activin type IIA receptor double mutant null mice formed tumours within the same time frame as the inhibin-α null mice. Histologically, the tumours in the double mutant were similar to the

single mutants. None of the double mutant mice developed the cancer cachexia-like syndrome, which significantly increased the animals life-span. Over 95% of female inhibin-α null mice were dead by 17 weeks of age, while 14 of 16 double mutant mice were alive at 18 weeks. Twelve of twelve compound null males lived past 15 weeks, while 95% of the inhibin-α null males died by 12 weeks of age. The majority of the livers showed no pathological findings, either grossly or histologically. However, one male and one female exhibited tremendous liver weight gain (2.3–2.5 g) and showed foci of dilated sinusoids without any concomitant venous sclerosis or necrosis. These changes are consistent with peliosis and could be the result of the high circulating estrogens found in the inhibin-α null mice although more likely secondary to other growth factors secreted from the tumours in rare cases. The compound null mice also had stomachs that were histologically similar to wild-type and activin type IIA receptor null mice. Endocrine profiles indicated that the activin levels were also elevated in the double mutant mice in comparison to wild-type controls, and even slightly elevated in comparison to the inhibin-α null mice.

These studies indicate that in the inhibin-α null mice, the signalling of activins through the activin type IIA receptor is directly responsible for the cancer cachexia-like syndrome present in these mice but not the formation of the gonadal tumours. This result is consistent with the results of ovary transplant studies discussed earlier (Matzuk *et al.* 1996).

11.11 Inhibin-α/Mullerian Inhibiting Substance Knockout Mice

Mullerian inhibiting substance (MIS) was originally discovered in males as the signal inducing the prenatal regression of the mullerian ductal system (a precursor to the oviduct, uterus and proximal vagina). MIS production is limited to Sertoli cells of the pre- and postnatal testes and granulosa cells of the postnatal ovary (Cate *et al.* 1990). In both males and females, the postnatal function of MIS is unknown, but the protein is believed to have anti-proliferative effects in the gonad. Transgenic female mice over-expressing MIS were born without uteri and exhibited ovarian regression a few weeks

after birth (Behringer *et al.* 1990). When the MIS gene was deleted in mice, males were found to have uteri and oviducts, and in a minority of mice (27%), Leydig cell hyperplasia was found (Behringer *et al.* 1994). These latter results suggested that MIS is a regulator of gonadal cell proliferation, similar to the inhibin.

When mice deficient in MIS and inhibin-α were generated, their phenotype displayed properties consistent with these two proteins acting synergistically to maintain a proper balance of growth in the testes (Matzuk *et al.* 1995b). These double mutant mice developed sex cord-stromal tumours and a cancer cachexia-like syndrome similar to the inhibin-α null mice. Males exhibited uteri, oviducts and a proximal vagina as expected from the loss of MIS. Of interest, the uterus in these double mutant mice was swollen to an extreme degree so that males exhibited an almost pregnancy-like abdominal girth. The elevated levels of estrogens in these mice was believed to play a role in this enlargement, and the distension was relieved when a pure anti-estrogen (i.e. ICI 182 780) was administered to these mice. However, when estrogens were administered to MIS null males, fluid accumulation was not found, indicating that other factors might be mitigating this effect. The testicular tumours in the double mutant mice were different than the tumours found in the inhibin-α null males. The tumours in the double mutants were larger, were derived from additional cell types, and were initiated at a much earlier age. The double mutant tumours were composed of the sex cord-stromal derivatives similar to those found in the inhibin-α null mice but were seen at an earlier age. In addition, tumours in the double mutant mice also contained neoplastic Leydig cells. Remarkably, by seven days, histologic examination showed the presence of small focal nests of neoplastic interstitial cells (the two individual mutant strains are essentially normal at this stage). By three weeks of age, there was a neoplastic proliferation of interstitial cells accompanied by hemorrhagic sex cord-stromal tumours (the inhibin-α null mice have only very small tumours by four to five weeks). Thus, when both MIS and inhibin were absent, there is an acceleration of tumourigenesis, suggesting that MIS and inhibin act in tandem to control growth of the Sertoli and Leydig cells in the testes.

11.12 Inhibin-α/MIS Receptor Knockout Mice

MIS transduces its signal similar to other TGF-β family members. A putative type II receptor for MIS was cloned in several species (Baarends *et al.* 1994, di Clemente *et al.* 1994, Imbeaud *et al.* 1995, Teixeira *et al.* 1996). Expression of the receptor protein and/or mRNA were found in mesenchymal cells underlying Mullerian duct epithelia during embryogenesis, along with the granulosa and Sertoli cells of foetal and adult gonads, and the gravid uterus (Baarends *et al.* 1995a, Baarends *et al.* 1995b, Baarends *et al.* 1994, di Clemente *et al.* 1994, Teixeira *et al.* 1996). Mishina and co-workers characterised the genomic organisation of the mouse MIS type II receptor, and by using ES cell technology, they created MIS type II receptor null mice (Mishina & Behringer 1996, Mishina *et al.* 1996).

These MIS type II receptor null mice phenocopied the MIS ligand null mice. Female mice lacking a functional MIS type II receptor are essentially normal, while males are internal pseudohermaphrodites. These MIS type II receptor null males possess an intact male reproductive tract, but also have a uterus and oviducts. They express the MIS ligand, but target tissues are hormone-insensitive. Spermatogenesis is functional, but the presence of the uterus in these males blocks sperm transport, rendering most of these mice infertile. Seminiferous tubules exhibit focal atrophy, while Leydig cells undergo hyperplasia that begins as early as two months of age. When crossed into the inhibin-α null background, the inhibin-α/MIS receptor double mutant mice (Mishina *et al.* 1996) exhibit the same characteristics as the inhibin-α/MIS null mice (Matzuk *et al.* 1995a). The inhibin-α/MIS receptor null females were essentially identical to the inhibin-α null females, while the double mutant males possessed a more complex phenotype. The inhibin-α/MIS receptor null male mice had large fluid filled uteri along with testicular tumours. Like the inhibin-α/MIS null males, the testicular tumours in the inhibin-α/MIS receptor null mice were composed of multi-focal sex cord-stromal cells along with multifocal sites of Leydig cell neoplasia. This data further substantiates the idea that inhibin and MIS act synergistically to influence the proliferative status of the testis.

11.13 Hypogonadal/Inhibin-α Knockout Mice

The hypogonadal (*hpg*) mouse is a strain of mouse that does not have a functional GnRH gene, leading to decreased serum levels of gonadotropins and infertility (Cattanach *et al.* 1977, Mason *et al.* 1986a). Both male and female *hpg* mice have gonads considerably smaller than wild-type mice. Females have altered ovarian function and do not undergo folliculogenesis past the secondary follicle stage. Males have seminiferous tubules of a small diameter with a block of spermatogenesis and possess poorly distinguishable Leydig cells. The most obvious mitigating factor in this phenotype is the low levels of gonadotropins. By crossing these mice into the inhibin-α null background, the effect of gonadotropins on tumourigenesis and tumour growth could be evaluated in the inhibin-α null mice. Indeed, gonadotropins have been implicated in gonadal tumourigenesis in several other cases. In transgenic mice, over-expression of an LH analog (LH-CTP) leads to ovarian tumours, while chronic hCG treatment of male rats resulted in Leydig cell tumours (Neumann 1991, Risma *et al.* 1995). SWR and SWJX recombinant inbred strains of mice develop heritable granulosa cell tumours at the onset of puberty (Beamer *et al.* 1993, Tennent *et al.* 1993). Elevated levels of FSH are associated with the development of ovarian cancer in elderly women (Godwin *et al.* 1992). Finally, the inhibin-α null mice show increased serum FSH while developing gonadal tumours (Matzuk *et al.* 1992). By crossing the *hpg* mice into the inhibin-α null background, a greater understanding of the role of gonadotropins in tumourgenesis could be ascertained.

The *hpg*/inhibin-α null mice were found to have a phenotype that is much less severe than the inhibin-α null mice alone (Kumar *et al.* 1996). These mice did not exhibit the cancer cachexia-like syndrome or the shortened life span found in the inhibin-α null mice. Females had small non-tumourous, non-haemorrhagic ovaries similar to the *hpg* females. Histologic examination of these ovaries revealed that follicular development did not extend past the pre-antral follicle stage. The only aberrent finding was the presence of seminiferous tubule-like structures that are also present in the inhibin-α null mice. These structures were found at all ages in the *hpg*/inhibin-α null females. The *hpg*/inhibin-α null males had small non-tumourous and non-hemorrhagic testes similar to *hpg* males. Microscopically, these mice had

testes similar to the *hpg* males except for the unexplained presence of a PAS-positive secretion in the lumen of the seminiferous tubules. Spermatogenesis was blocked at the same stage as the *hpg* mice, and there was similar hypoplasia of Leydig cells. Examination of the adrenal glands did not reveal any pathologic findings.

These studies show that gonadotropins are essential modifiers of tumourgenesis in the inhibin-α null mice. The lack of tumour formation and concommitant wasting syndrome in the *hpg*/inhibin-α null mice is evidence that LH and FSH play a definitive role in the progression and maintenance of the gonadal tumours found in the inhibin-α null mice. Thus, the arrest of tumourigenesis in the pre-malignant stages may be a function of the proliferative state of the granulosa/Sertoli cell. The lack of inhibins in the *hpg*/inhibin-α null mice may make the granulosa/Sertoli cells prone to malignant transformation, but the lack of a stimulus in the form of gonadotropins directly slows the tumourigenesis process. Another possibility is that the lack of gonadotropins indirectly inhibits some other compound from being expressed (e.g. androgens) which is the tumourigenic factor in these mice. In summary, the lack of gonadotropins leads to a block in the formation of tumours in inhibin-α null mice.

11.14 tfm/Inhibin-α Knockout Mice

The role that androgens play in the phenotype of inhibin-α null mice was also investigated (Shou *et al.* 1997). As previously stated, SWR mice are prone to developing granulosa cell tumours. In ovarian transfer experiments, this tumourigenesis required gonadotropins. Of interest was that hCG, but not FSH, could induce these tumours. Dihydroepiandrosterone, an androgen precursor, was also found to induce tumours implicating hCG's steroidogenic capabilities in the formation of ovarian tumours (Beamer *et al.* 1993, Tennent *et al.* 1993). This data suggests that androgens may play a role in gonadal tumourigenesis. To determine that role, inhibin-α null mice were bred into the testicular feminisation mouse background (*tfm* mice).

tfm mice have an X-linked mutation in the androgen receptor gene rendering these mice insensitive to androgens (Charest *et al.* 1991, He *et al.* 1991). Phenotypically, male *tfm* mice are born with the secondary sex characteristics of females. In addition, their testes are small and undescended with spermatogenesis blocked at meiotic prophase. This mouse line was bred into the inhibin-α null background, to generate double mutant male mice. These double mutant male mice undergo gonadal tumourigenesis and exhibit the wasting syndrome displayed in the inhibin-α null mice, although the nature of these pathologies was less severe. Analysis of body weight revealed that the initiation of the wasting syndrome was similar to the inhibin-α null mice, and the weight curves paralleled each other although *tfm* males start at a lower body weight than inhibin-α null males (i.e. their body weight was more similar to females). Surprisingly, these double mutant mice displayed an increased longevity in comparison to the inhibin-α null mice alone. Approximately half of the compound mutant males lived until 17–23 weeks of age in comparison to the inhibin-α null males which have a 95% mortality rate by 12 weeks. Histologic data revealed that at five weeks of age, the double mutants formed tumours that were multifocal and non-haemorrhagic. Indeed, the double mutant mice seemed to have tumours that suggested a hyperplastic state, in comparison to the inhibin-α null mice where the tumours were focal and haemorrhagic. As the compound mutant mice aged, 65% never formed haemorrhagic lesions, implying that these mice had lesions that were less traumatic or invasive. Activin levels were unaffected by the loss of androgen sensitivity showing that the tumours were reacting to the loss of the androgen signal and not a decreased cachexia-inducing signal.

Thus the loss of androgen sensitivity in the inhibin-α null background seems to either lower the local invasiveness and concommitant trauma of the sex-cord stromal tumours, or perhaps lower the angiogenic potential of the tumours. Thus, in contrast to the gonadotropins, androgens do not seem to be essential for the progression of tumourigensis in the inhibin-α null mice but seem to play a modifying role in the initiation and progression of tumourigenesis in mice deficient in inhibin.

11.15 Conclusions

Inhibin and activin have been shown through ES cell technology to have an impact on many different stages of development and life of mice. The lack of ActRIB blocks gastrulation and halts embryogenesis. Mutations in the ActRIIB, activin/inhibin β_A-subunit, and activin/inhibin β_B-subunit led to defects in later stages of development that in all three cases caused some postnatal lethality. Reproductive deficits were reported where the inhibin-α, activin/inhibin β_B-subunit and ActRIIA genes were mutated. Finally, the loss of inhibin-α led to a phenotype where reproduction was blocked and the gonads underwent tumourigenesis that led to death in older animals. Combinations of these and other mutant backgrounds were subsequently used to further understand the events that led to each phenotype.

Suprisingly, only one of these mutant null mice caused embryonic lethality. Despite the early expression of the activin and their numerous receptors, only the activin type IB receptor blocked development in its early stages and was embryonically lethal (Gu *et al.* 1998). The loss of ActRIB disrupted the epiblast of the developing embryo, blocking gastrulation and subsequent development. This defect was rescued by the expression of a human ALK4 transgene. The loss of the activin/inhibin β_A-subunit led to phenotype expressing a postnatal lethality (Matzuk *et al.* 1995d). These mice were born without whiskers, incisors or mandibular molars. They also expressed various palatal malformations, which led to their inability to suckle and subsequent death. Compound activin β_A/β_B null mice died in a similar manner, although some of these mice were born with their eyes open (a defect attributed to the loss of activin-β_B) (Matzuk *et al.* 1995c). The loss of the activin type IIA receptor had a low penetrant postnatal lethal phenotype (Matzuk *et al.* 1995c). This lethality was manifested by similar craniofacial defects, rendering them unable to suckle. Other expressed malformations were the loss of incisors, defective eyelids, micrognathia and defects in Meckel's cartilage. Finally, mice without activin type IIB receptors expressed a phenotype that was different from these others. Mice were born alive, but succumbed to severe cardiovascular malformations within hours of birth

(Oh & Li 1997). These mice also exhibited a loss of lateral asymmetry and alterations in axial skeleton formation consistent with many human congenital deformities. All of these proteins (i.e. activin ligands and ActRIIA and ActRIIB) are expressed early in development, yet only the ActRIB mutation was lethal *in utero*. This data indicates that there may be a large degree of redundancy in these developmental pathways or may indicate that expression may not necessarily define function or importance during the early moments of development.

Several of these mutant mouse lines had defects in their reproduction and fertility. Loss of activin type IIA receptors caused some postnatal lethality in mice (as discussed previously), but these mice were predominantly defective in their reproductive potential (Matzuk *et al.* 1995d). Males exhibited delayed fertility, while females never attained reproductive maturity. These mice were even more interesting because of their apparent similarities with the FSH null mice (Kumar *et al.* 1997). Mutations in the activin/inhibin β_B-subunit led to reproductive deficits of a different nature (Schrewe *et al.* 1994, Vassalli *et al.* 1994). Males were grossly normal, but females lost their litters in many cases because of defects in parturition and/or their inability to nurse their pups. These mice also expressed increased serum FSH. Inhibin-α null males had a short period of fertility early in their lives, but they eventually developed testicular tumours that blocked this ability (Matzuk *et al.* 1992). Superovulation studies showed that inhibin-α null females were defective at ovulation (Matzuk *et al.* 1996). Loss of inhibin may alter the capacity of these females to cycle, rendering them infertile. Tumour development further decreases the chances of pregnancy. Thus, as expected by their known functions in reproduction and fertility, mice lacking β_B, ActRIIA and inhibin-α were found to be defective in their reproductive ability.

The formation of tumours in inhibin-α null mice was a somewhat unexpected result. The lack of inhibin caused the granulosa or Sertoli cells in these mice to undergo malignant transformation and form haemorrhagic tumours (Matzuk *et al.* 1992). These tumours secreted high amounts of activin that led to a cancer cachexia-like syndrome. High levels of activin

in the serum was hepatotoxic and also led to the loss of parietal cells from the stomach (Matzuk *et al.* 1994). This wasting syndrome eventually led to the death of 95% of the males by 12 weeks and 95% of the females by 17 weeks of age. Castrated inhibin-α null mice lived longer before the onset of the wasting syndrome, but they also died of its effects. Upon autopsy, these mice were found to have formed adrenal tumours that also secreted high amounts of activin. When the inhibin-α null mice were bred into the ActRIIA null background, tumours formed but the wasting syndrome did not occur confirming the role of activin in its etiology (Coerver *et al.* 1996). Other compound inhibin-α mutant mice were generated. The loss of inhibin and MIS or the MIS type II receptor led to a more aggressive tumourigenesis with an additional Leydig cell component (Matzuk *et al.* 1995a, Mishina *et al.* 1996). Mice lacking inhibin and a functional androgen receptor had less aggressive tumours, notable for their decreased haemorrhagic nature (Shou *et al.* 1997). Finally, when inhibin-α null mice were bred into the *hpg* mutant background, tumourgenesis was substantially inhibited (Kumar *et al.* 1996). Double mutant females were similar to *hpg* controls except for the presence of "Sertoli-only" tubules in the ovary. Double mutant males were also similar to *hpg* controls outside of the presence of a PAS-positive substance found in the lumen of the seminiferous tubules. These results point to a tumour suppressor function for inhibin. The formation of tumours in mice lacking inhibin is modified by the presence or absence of several factors leading to either more or less aggressive malignancies.

Lastly, this chapter documents the phenotypes produced upon mutation of proteins belonging to the activin and inhibin signal transduction pathways. The importance of these proteins is evident by the phenotypes generated by their absence in mice. More fundamental studies concerning these pathways are underway by generating mice deficient in other proteins important in activin and inhibin signalling and by producing additional double mutant mouse lines.

11.16 Summary

Mutation(s)	Phenotype
• Inhibin-α (Intact)	Gonadal tumours; cancer cachexia-like syndrome secondary to tumourigenesis; majority die by 17 weeks of age.
• Inhibin-α (Castrate)	Adrenal tumours after 21 weeks; cancer cachexia-like syndrome secondary to tumourgenesis; median death by 33 weeks of age.
• Activin/inhibin β_A	Postnatal lethality; lack of whiskers, incisors mandibular molars and palatal defects.
• Activin/inhibin β_B	Hybrid background mice born with eyes open; females have an extended gestation period and were unable to nurse properly; increased serum FSH.
• Activin/inhibin β_A/ Activin/inhibin β_B	Additive defects of β_A and β_B mutations.
• Activin Type IB Receptor (ActRIB)	Embryonic lethality; development halted at the pre-gastrulation stage.
• Activin Type IIA Receptor (ActRIIA)	Partial postnatal lethality with cranio-facial deformities; viable adult males exhibit delayed fertility, and females are infertile due to a block at the antral follicle stage.
• Activin Type IIB Receptor (ActRIIB)	Postnatal lethality; severe cardiovascular defect; loss of lateral asymmetry, and axial skeletal defects.

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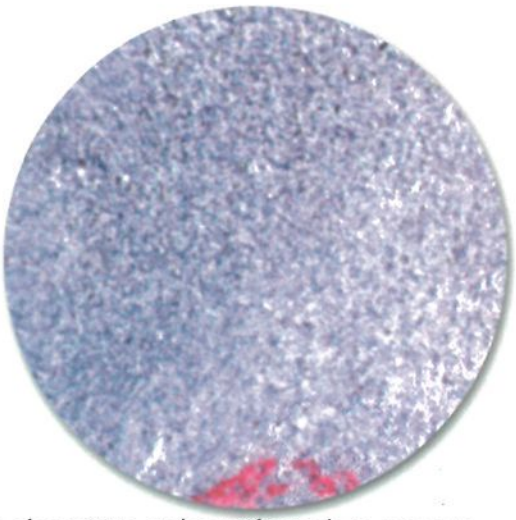
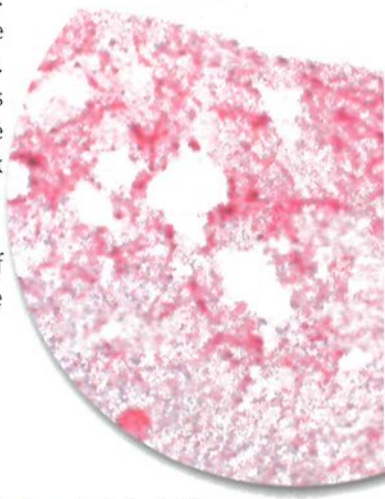
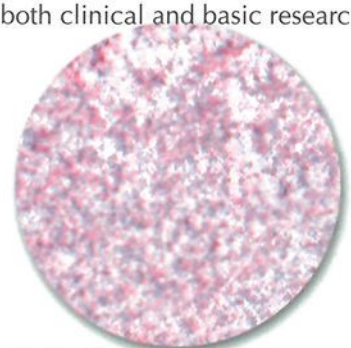


Development of sensitive and specific tools to measure inhibins and activins in serum and biological fluid within the last decade has provided illuminating insights into the physiology and pathology of human gonadal function. Rapid progress has been made in both reproductive physiology and possible applications in clinical practice. In pregnancy, the discovery that these placental hormones are altered in disease states, such as pre-eclampsia, have proven to be one of the most exciting aspects of work in this area of human biology.

This book comprehensively reviews the current state of knowledge in this field and identifies areas for future work in both clinical and basic research.





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