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The Biology Of Activin: Recent Advances In Structure, Regulation And Function

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Abstract

Activin was discovered in the 1980's as a gonadal protein that stimulated FSH release from pituitary gonadotropes and was thought of as a reproductive hormone. In the ensuing decades many additional activities of activin were described and it was found to be produced in a wide variety of cell types at nearly all stages of development. Its signaling and actions are regulated intracellularly as well as by extracellular antagonists. Over the past 5 years a number of important advances have been made that clarify our understanding of the structural basis for signaling and regulation, as well as the biological roles of activin in stem cells, embryonic development, and in adults. These include the crystallization of activin in complex with the activin type II receptor ActRIIB, or with the binding proteins follistatin and follistatin-like 3 (FSTL3), and identification of the activin roles in gonadal sex development, follicle development and luteolysis, in β-cell proliferation and function in the islet, in stem cell self-renewal and differentiation into different cell types, and in immune cells. These advances are reviewed to provide perspective for future studies.

Inhibin and activin were purified from gonadal fluids based on their ability to inhibit or stimulate (respectively) release of FSH from cultured pituitary gonadotropes. Following purification and molecular characterization, these hormones were identified as members of the TGFβ superfamily, which now includes more than 40 ligands including TGFβ, inhibin, myostatin and bone morphogenetic proteins (BMPs)(Vale *et al.* 1988). Activin receptors were discovered later by functional cloning, revealing a type II receptor with serine/threonine kinase activity as the binding moiety (Mathews & Vale 1993), which was later found to phosphorylate a type I receptor after ligand (Tsuchida *et al.* 2008). These discoveries, along with the identification and characterization of extracellular activin antagonists such as follistatin (FST) and follistatin like 3 (FSTL3) (Welt *et al.* 2002), spurred a torrent of research activity into activin's biological roles in a wide spectrum of tissues and systems ranging from fate determination in embryos to homeostatic mechanism in adults (reviewed in (Mather *et al.* 1992; Mather *et al.* 1997; DePaolo 1997; Welt *et al.* 2002)). One of the continuing challenges in this field has been to decipher the *in vivo* activities of ligands that act primarily through autocrine and paracrine mechanisms where the value of the more classical endocrinological ablation and replacement paradigms is limited. Moreover, the requirement for activin during development has restricted utilization of global knockout technology to address this issue. The more recent advent of conditional knockout technology, regulatable expression systems, and

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siRNA suppression technology is opening the door for more detailed investigations of activin's tissue-specific roles in adults.

The basics of activin signaling have been known for some time, although emerging details of cross-talk with other signaling pathways and an increasingly complex web of intracellular regulators adds new wrinkles at a regular pace (reviewed in (Tsuchida *et al.* 2008). This review will first focus on recent advances in our understanding of the structural basis for activin's interactions with its receptors and antagonists along with new information about how activin signaling and biosynthesis are regulated, after which we will summarize advances in our understanding of activin's biological roles, primarily in adults, published since our last comprehensive review (Welt *et al.* 2002). The role of activin in endocrine cancer, while important, was too vast to be included in this review.

A. Advances in activin structure-function relationships

1. Activin-receptor crystal structures

The canonical activin signaling pathway involves an activin dimer binding to one of two type II receptors (ActRIIA or ActRIIB) in complex with activin's type I receptor ActRIB (a. k. a, activin receptor-like kinase 4, Alk4), which ultimately leads to phosphorylation of the type I receptor. This receptor then phosphorylates the second messenger molecules Smad2 and Smad3 that, once activated, complex with a common Smad, Smad4. This complex then translocates to the nucleus where it activates gene transcription. Smad7 was identified as an inhibitory Smad that blocks phosphorylation of Smads 2 and 3, thereby providing a short loop negative feedback regulation for this signaling pathway (reviewed in (Tsuchida *et al.* 2008)). Another level of regulation occurs extracellularly through soluble antagonists like follistatin and FSTL3 (Schneyer *et al.* 2001) or membrane bound modifiers like BAMBI (BMP and activin receptor membrane bound inhibitor) and Cripto (a GPI-anchored membrane protein, which inhibits activin signaling by forming an "inert" complex with activin and ActRIIA) (Kelber *et al.* 2008; Onichtchouk *et al.* 1999). Despite focused efforts, crystallization of activin alone was unsuccessful, suggesting that there may be considerable flexibility in how the two disulfide-linked subunits of activin are aligned. This was confirmed when activin A was crystallized in complex with ActRIIB extracellular domain (Thompson *et al.* 2003). In this structure, ActRIIB binds to the outer edges of the so-called finger regions on the activin dimer. Interestingly, the activin dimer in this model was tightly folded into a compact conformation unlike the extended configuration of other TGFβ family members previously crystallized (Hinck *et al.* 1996; Griffith *et al.* 1996). Activin's flexibility and compact conformation was confirmed in a more highly resolved crystal structure with ActRIIB and suggested that this movement allowed binding sites for the type I receptor to be exposed once the type II receptor sites were occupied, thereby suggesting a mechanism for how the hexameric complex consisting of the activin dimer with two type II and two type I receptors is organized and activated (Greenwald *et al.* 2004). Support for this model comes from the remarkable achievement of crystallization of the ternary signaling complex comprised of a related ligand (BMP-2) and both receptor subtypes (Allendorph *et al.* 2006). These results showed that the ligand is the central coordinator of the complex with an absence of direct contacts between the receptors themselves (Allendorph *et al.* 2006). Moreover, they show that receptor specificity depends on specific unique residues arranged around a conserved hydrophobic core on type I receptors (Allendorph *et al.* 2006).

2. Activin Antagonist crystal structures

Follistatin, originally identified and isolated from both bovine and porcine follicular fluids on the basis of its inhibition of pituitary FSH secretion, is a monomeric glycosylated polypeptide chain that binds activin with high affinity and neutralizes most but not all of its biological

actions. FSTL3 was cloned from a B cell leukemia line and it was originally named FLRG (FST-related gene) based upon primary sequence homology to follistatin. Subsequently, FSTL3 was also found to be a high affinity activin binding protein (reviewed in (6)).

As detailed previously, point mutagenesis and domain swapping studies were used to determine portions of the FST molecule that were critical for its neutralization of activin activity (Welt *et al.* 2002). FST is comprised largely of three successive 73–77 residue, 10-cysteine follistatin domains (FSDs), preceded by a 63-residue N-terminal domain (Welt *et al.* 2002). Residues on FSD2 and on the N-domain were found to be critical for activin binding (Sidis *et al.* 2005; Keutmann *et al.* 2004; Sidis *et al.* 2001). Moreover, the N-domain, followed by at least 2 other FSDs were required for activin binding and neutralization (Keutmann *et al.* 2004).

Rearrangement or substitution among the FSDs impaired or abolished activin binding, indicating that the number and order of FST domains were important for full activity (Keutmann *et al.* 2004). While these observations were instructive and consistent with binding to proposed receptor binding surfaces on activin, it was not until FST was crystallized with activin A that the significance of these observations were fully appreciated (Thompson *et al.* 2005). Two FST molecules envelop the activin dimer, covering a large percentage of the surface and completely blocking both type I and type II receptor binding sites (Thompson *et al.* 2005). Moreover, it appeared that the C-terminus of one FST molecule contacted the N-terminus of the other FST molecule as if to lock them in place (Thompson *et al.* 2005), suggesting a possible mechanism to account for the nearly irreversible kinetics identified much earlier (Schneyer *et al.* 1996; Schneyer *et al.* 1994). The FST-Activin structure also confirmed the importance of FSD2 in contacting a large portion of the activin surface, thereby contributing substantially to the overall affinity of the interaction (Thompson *et al.* 2005; Keutmann *et al.* 2004).

More recently, FSTL3, which has one FSD fewer than FST, has been crystallized in complex with activin A revealing a similar overall structure with two FSTL3 molecules surrounding the activin dimer (Stamler *et al.* 2008). However, in this case, the N-domain of FSTL3 contacts activin more extensively than in FST, enhancing its importance to activin binding and perhaps even compensating for the absence of a third FSD in FSTL3 (Stamler *et al.* 2008). The N-domain contacts may also influence ligand binding specificity (Stamler *et al.* 2008), thereby accounting for the previously determined difference in BMP binding between FST and FSTL3 (Sidis *et al.* 2006).

Myostatin is also a member of the TGFβ superfamily closely related to activin, and follistatin can bind myostatin with relatively high affinity, although not as high as activin itself (Sidis *et al.* 2006). Through site directed mutagenesis, it was found that mutations in FSD2, or replacement of FSD2 with FSD1 reduced activin binding but maintained myostatin binding activity whereas mutations in FSD1 or replacement of FSD1 with another copy of FSD2 diminished myostatin binding but not activin binding (Schneyer *et al.* 2008). Thus, it was possible to create a myostatin-selective analog of FST that may be useful for designing new treatments for muscle wasting disorders (Schneyer *et al.* 2008).

3. Regulation of activin activity by antagonists

Although it has been known for years that FST is produced as three isoforms resulting from alternative splicing and post-translational proteolytic processing (Sugino *et al.* 1997), the relative activities of these different isoforms remained to be fully elucidated. As reviewed previously (Welt *et al.* 2002), the shorter FST288 isoform has a higher affinity for heparin-sulfated proteoglycans located on the cell surface allowing FST288 to be concentrated at the outer surface of the plasma membrane and potentially prevent action of autocrine acting activin in addition to activin arriving from other cells or tissues (i.e., paracrine or endocrine acting

activin). On the other hand, the longest isoform, FST315, was localized primarily in the circulation (Schneyer *et al.* 2004), consistent with its reduced affinity for heparin in the unliganded state. The intermediate isoform, FST303, was found primarily in gonadal extracts and fluids and thought to be derived by proteolytic processing of the C-terminal acidic tail (Sugino *et al.* 1997). FSTL3, which shares a number of structural features with FST, does not contain a heparin binding sequence and thus, does not bind cell-surface proteoglycans under normal conditions (Sidis *et al.* 2002). These biochemical distinctions suggested that the different FST isoforms and FSTL3 had distinct biological actions *in vivo* (Sidis *et al.* 2002; Schneyer *et al.* 2001; Tortoriello *et al.* 2001). This hypothesis was tested by producing recombinant FST isoforms and assessing their binding kinetics for different TGFβ ligands as well as their ability to inhibit biological activity when added as proteins (simulating paracrine/endocrine actions) or when transfected into cells (simulating autocrine actions). Activin binding kinetics were comparable between the FST isoforms and FSTL3 but cell-surface binding differed markedly (Sidis *et al.* 2006) as expected from previous studies. Interestingly, inhibition of endogenous (e.g., autocrine) activin activity correlated closely with surface binding activity, whereas inhibition of exogenous (paracrine or endocrine) activin was not significantly different among the FST isoforms, consistent with their similar binding affinities (Sidis *et al.* 2006). Expression of FST315 as a membrane-anchored protein transformed it from a soluble with weak antagonism to autocrine activin into an isoform that inhibited autocrine activin activity equal to that of the FST288 isoform (Sidis *et al.* 2006), supporting the concept that membrane localization via the heparin binding sequence significantly increases inhibition of autocrine-acting activin. In addition, myostatin and BMP 6 and 7 were inhibited by the FST isoforms, albeit to a lesser degree than that of activin, and this inhibition also correlated with their cell-surface binding (Sidis *et al.* 2006). These results support the hypothesis that differential cell-surface binding is the primary determinant of FST isoform biological activity and that these isoforms may have distinct bioactivities *in vivo*.

In support of this latter hypothesis, human FST 315 or FST288 cDNAs were expressed transgenically under control of the human promoter and crossed into the FST null mouse line (Lin *et al.* 2008), which had been previously demonstrated to be neonatally lethal (Matzuk *et al.* 1995c). While the FST288 expressing TG mice did not rescue this lethality, the FST315 expressing construct allowed mice to survive to adulthood with partial reversal of the skeletal abnormalities seen in FST-null mice (Lin *et al.* 2008). However, these mice exhibited neonatal growth retardation, impaired tail growth and female infertility that may have resulted from failure of corpus luteum formation and a decline in ovarian follicle population (Lin *et al.* 2008). These results suggest that the FST288 isoform may be required for normal vascularization and ovarian function whereas the FST315 isoform is sufficient for other regulatory actions that when absent, result in neonatal death (Lin *et al.* 2008), consistent with isoform-specific actions of FST *in vivo*. More detailed analysis awaits production of mice capable of producing individual FST isoforms from the endogenous FST locus.

Biochemical differences among the FST isoforms and FSTL3 also govern their biosynthesis and secretion. Using ^{35}S pulse-chase labeling, FST315 was found to be synthesized and secreted the fastest while FST288 was secreted more slowly, with some FST288 remaining within the cell after extended incubation (Saito *et al.* 2005). FSTL3 was the slowest to be secreted with newly synthesized proteins being both secreted and transported to the nucleus (Saito *et al.* 2005). Interestingly, this nuclear FSTL3 was glycosylated but not to the same degree as secreted FSTL3, suggesting it may be derived from an ER-resident pool that is transported directly to the nucleus without first being secreted (Saito *et al.* 2005). These results indicate that both FST and FSTL3 may have both intracellular and extracellular roles that are governed to some degree by heparin-binding affinity (Saito *et al.* 2005).

4. Regulation of activin synthesis

Inhibin and activin share a common β-subunit with activin constituting a dimer of two β-subunits whereas inhibin contains a related α-subunit linked to the β-subunit (Vale *et al.* 1988). Thus, regulation of α-subunit biosynthesis and dimerization with the β-subunit can alter activin biosynthesis in cells that synthesize both subunits such as ovarian granulosa cells. The mature portion of the α-subunit is glycosylated and elimination of the carbohydrate chain at asp 268 inhibited inhibin secretion without altering activin secretion, while mutations of the β-subunit that induced its glycosylation also favored inhibin production (Antenos *et al.* 2007). These observations indicate that the degree of glycosylation determines the relative proportion of inhibin and activin produced in such cells (Antenos *et al.* 2007). Activin also stimulated production of both α-and β-subunit mRNA and protein, as well as the required proteolytic processing that removed mature hormone from the precursor portions of the subunit (Antenos *et al.* 2008). Interestingly, activin also stimulates furin biosynthesis through a smad2/3-dependent process, suggesting the presence of a short feedback loop to enhance secretion of mature activin and inhibin (Antenos *et al.* 2008).

B. Advances in biological actions of activin in adults and embryonic stem cells

1. Activin and reproduction

Biological roles for activin have been proposed in a number of reproductive organs including the gonads, uterus and pituitary, where it regulates processes such as folliculogenesis, spermatogenesis, and pregnancy (reviewed in (Welt *et al.* 2002)). Over the past several years, significant progress has been made in delineating the roles and mechanisms of activins in embryonic ovary development, follicle development, luteolysis and testis development, as well as in regulation of FSHβ gene transcription.

1.a. Activin and Gonadal Sex Determination—Emerging evidence suggests that follistatin and activins influence differentiation of the indifferent gonad as well as the early stages of folliculogenesis. Embryonic XX, but not XY gonads express FST, with mRNA becoming detectable in the E11.5 XX mouse gonad (Menke & Page 2002; Yao *et al.* 2004). In support of a role for FST in regulating development of female gonads, FST null XX gonads develop a coelomic vessel, the rudiment of the testis-specific vasculature structure that organizes and patterns the developing testis, suggesting that follistatin possesses an anti-testis function (Yao *et al.* 2004). In the mouse XX gonad on E12.5, βB but notβA subunit mRNA is expressed, which is in contrast to the expression of both βB and βA subunit mRNA in XY gonads. Interestingly, in FST and βB subunit double knockout embryos, vasculature development in XX gonads was reversed from the FST null phenotype to wild type (i.e., female) while local application of activin B induced ectopic formation of coelomic vessel in cultured XX gonads, and βB knockout XY gonads exhibited defects in coelomic vessel formation (Yao *et al.* 2004). These results suggest that activin B induces the formation of the coelomic vessel in both XY and XX gonads, but this action is normally inhibited by follistatin in the XX gonad (Yao *et al.* 2004; Yao *et al.* 2006a). Although the role of activin and follistatin in gonadal differentiation has not been established in other species, Xia et al., found that FST concentrations were higher in fetal blood and allantoic fluids from female fetuses compared to male fetuses during early pregnancy in sheep (Xia *et al.* 2008b), suggesting that FST may also have important roles in sexual development in non-rodent species.

1.b. Activin and Follicle Development—Initial follicle pool formation is critical for normal fertility in adult female mammals. Studies on developing mouse and human ovaries suggest that activins are involved in regulation of germ cell proliferation during the developmental period leading up to primordial follicle formation, which occurs at 24 hr after

birth in the mouse and 21 weeks of gestation in the human (Bristol-Gould *et al.* 2006; Martins da Silva *et al.* 2004). In mouse ovaries from postnatal days 0 and 4, activin βA and βB subunits, and activin receptors ActRIIA, ActRIIB and ALK4 are expressed in both somatic cells and oocytes, suggesting that neonatal mouse ovaries produce activin and are activin-responsive (Bristol-Gould *et al.* 2006). Administration of activin A to female pups from birth to day 4 increased germ cell and granulosa cell proliferation, as well as the number of primordial follicles formed from the germ cell nests, without any effect on apoptosis in these cells (Bristol-Gould *et al.* 2006). Activin subunits, βA and βB, the receptors ActRIIA and ActRIIB, and downstream effectors Smad2/3 are also expressed in human fetal ovaries at 14–19 weeks of gestation (Martins da Silva *et al.* 2004; Coutts *et al.* 2008). Interestingly, βA subunit mRNA expression increased 2-fold from 14 to 19 weeks of gestation, and treatment of human ovaries between 14 and 17 weeks of gestation with activin A dramatically increased oogonial proliferation (Martins da Silva *et al.* 2004). These results suggest that activin signaling may play an important role in determining the size of the primordial follicle pool in humans as well as mice. Since this is the total endowment of follicles for the female's lifetime, these results also suggest that activin signaling may be important in premature ovarian failure where functional follicles are prematurely exhausted (Anasti 1998).

Activins and follistatins were originally isolated from follicular fluids and these proteins are predominantly expressed in granulosa cells. Multiple roles have been proposed for granulosa cell activin including regulation of proliferation, differentiation and steroidogenesis (reviewed in (Welt *et al.* 2002)). More recently discovered actions for activin include synergistic actions of activin with FSH to promote G1/S transition and cell proliferation in rat primary granulosa cells, a process accompanied by induction of CycD2 expression and phosphorylation of retinoblastoma protein (Rb) (Ogawa *et al.* 2003; Park *et al.* 2005). In addition, induction of CycD2 by activin/FSH involved activation of phosphatidylinositol 3- kinase (PI 3-kinase) and Akt pathways (Park *et al.* 2005). Activin was also shown to increase the estrogen receptors αand β (ERα and ERβ) in mouse granulosa cells both *in vitro* and *in vivo* suggesting that the activin and estrogen signaling pathways intersect to regulate granulosa cell activity and follicle development (Kipp *et al.* 2007).

Genetic elimination of activin βA resulted in mouse pups that died within 24 hours of birth with craniofacial and other defects (Matzuk *et al.* 1995b) while activin βB deficient mice survived to become fertile adults but offspring of βB-deficient females died perinatally, perhaps from insufficient lactation (Vassalli *et al.* 1994). To explore the role of activin A in ovarian follicle development, granulosa cell-specific conditional βA subunit knockout mice were created, resulting in subfertility with enhanced corpus luteum (CL) survival and accumulation within the ovary (Pangas *et al.* 2007). These results, in combination with the observation that replacement of mature βA with mature βB results in subfertility (Brown *et al.* 2000), suggest that both activins A and B have important roles within the ovary with activin A being functionally dominant over activin B (Pangas *et al.* 2007).

Transgenic overexpression of FST in ovaries led to a blockade in follicular development before antral follicle formation suggesting FST had important roles in ovarian physiology (Guo *et al.* 1998), perhaps through regulation of activin action. Consistent with this observation, granulosa cell-specific overexpression of FSTL3, the related activin antagonist, resulted in fewer antral follicles in adult females (Xia *et al.* 2004), while deletion of FSTL3 resulted in mild subfertility in females and increased testis size in males (Mukherjee *et al.* 2007). Since global FST null mice died just after birth and are therefore not useful for analyzing adult FST roles, Jorgez et al (Jorgez *et al.* 2003) created a conditional *fst* allele and used these mice to delete FST production from ovarian follicles. This led to decreased antral follicle number in 3-month old mice, as well as decreased litter number and size (Jorgez *et al.* 2003), suggesting that optimized signaling of activins, and/or other TGF-β family members bound by FST and

FSTL3 (e.g. BMP6 and BMP7, see (Schneyer *et al.* 2008; Sidis *et al.* 2006)) are required to maintain normal development from secondary to antral follicles (Jorgez *et al.* 2003). An additional phenotype developed as the females matured with accelerated loss of follicles, early ovarian senescence and elevated gonadotropins (Jorgez *et al.* 2003) similar to the human infertility condition known as premature ovarian failure (Anasti 1998).

Several FST isoforms were recently identified in ovine (Xia *et al.* 2008a) and bovine gonads (Glister *et al.* 2006) by Western blotting. The relative amounts of these FST isoforms varied differentially during follicle maturation although the total FST concentration in both ovine and bovine follicular fluids declined with follicle development, suggesting that regulation of activin action by intrafollicular FST changes as follicles mature (Glister *et al.* 2006; Xia *et al.* 2008a). In fact, FST isoform distribution and biological role appears to depend on biochemical features distinct within the FST isoforms themselves such as the ability to bind to heparin sulfated proteoglycans on the surface of cells (Sidis *et al.* 2006). Thus, the longest isoform that has little cell-surface binding ability is largely found in serum (Schneyer *et al.* 2004) while follicular fluid appears to contain predominantly the intermediate sized isoform, FST303 (Sugino *et al.* 1993). This model is further supported by a mouse model where a human FST315 minigene was expressed transgenically under the direction of the human promoter and then crossed with the FST null mouse model described above (Lin *et al.* 2008). These FST315/303-expressing mice survived to adulthood but females were subfertile with reduced numbers of primordial, primary and secondary follicles, and no antral follicles and corpus luteum (Lin *et al.* 2008).

1.c. Activin and Luteolysis—Using cocultures of cells isolated from human corpus luteum (CL), Meyers et al demonstrated that activin derived from luteinized granulosa cells increased expression of matrix metalloproteinase-2 (MMP-2) by fibroblast-like cells, and that this activity was inhibited by FST (Myers *et al.* 2008; Myers *et al.* 2007). MMPs are key proteolytic enzymes involved in the degradation of the extracellular matrix, and MMP-2 is an important luteolytic agent during the demise of CL (Myers *et al.* 2007), thereby suggesting that activin may play an active role in regression of CL survival. This notion is supported by analyses of ovary-specific deletion of activins in mice in which the ovaries displayed progressive accumulation of CLs and the absence of CL regression (Pangas *et al.* 2007). Additional support for this concept comes from the observation that FST mRNA expression is dramatically reduced in regressing CLs compared to newly formed CLs in rats which created a low FST/high activin environment (Erickson & Shimasaki 2003). On the other hand, FST release from the ovary increased at luteolysis in sheep, which may be another mechanism to create a low FST environment through FST secretion rather than keeping the FST in follicular fluid (Xia *et al.* 2008b).

1.d. Activin and Testicular Function—Smaller testes and reduced fertility were observed in mice when activin signaling was down regulated by inactivation of ActRIIA (Matzuk *et al.* 1995a), or when FST (Guo *et al.* 1998) or FSTL3 (Xia *et al.* 2004) were transgenically overexpressed. Moreover, in the FSTL3 transgenic mice, reduced testis weight was observed as early as postnatal day 7 (Xia *et al.* 2004). The mechanism whereby activin might control testis size was suggested in early studies showing that activin can directly promote Sertoli cell proliferation in culture (Mather *et al.* 1990), and supported by more recent observations that the primary source for activin-A in the testis was peritubular myoid (PTM) cells which acts in a paracrine fashion to promote Sertoli cell proliferation only when the Sertoli cells were obtained between neonatal days 3–9 (Buzzard *et al.* 2003). Taken together, these results support the concept that disrupted activin signaling may limit Sertoli cell proliferation in a critical developmental window which persists as reduced testis weight in adults because the number of Sertoli cells established during this proliferative phase in neonates constitutes an upper limit to spermatogenic output in adults (Sharpe *et al.* 2003). In addition, activin signaling during

early testicular development appears to determine ultimate testis size and function in adults, and may be regulated by activin antagonists including FSTL3 (Xia *et al.* 2004) and FST (Guo *et al.* 1998).

1.e. Activin Actions in the Pituitary—Activin was originally purified on the basis of its ability to stimulate FSH release from pituitary gonadotropes (Vale *et al.* 1988) and it was subsequently shown that activin is a critical regulator of FSHβ biosynthesis (Weiss *et al.* 1992). More recent studies have shown that Smad3 is the principal regulator of activin-induced FSHβ subunit transcription, while Smad2 may be more involved in regulation of basal FSHβ transcription in murine immortalized LβT2 gonadotrope cells (Suszko *et al.* 2003; Bernard 2004; Lamba *et al.* 2006; Bernard 2004; Suszko *et al.* 2008; Suszko *et al.* 2005; Gregory & Kaiser 2004). This functional difference between Smad2 and Smad3 appears to be attributed to the second of the two loops in the Smad2 MH1 domain, which is thought to bury a β-hairpin loop structure thereby preventing Smad2 from directly binding its cognate DNA (Suszko *et al.* 2005). In addition, activin stimulation of FSHβ gene transcription involves Smad3 partners, including Pitx2c (a pituitary-specific transcription factor) (Suszko *et al.* 2008) and TALE homeodomain proteins Pbx1 and Prep1 (Bailey *et al.* 2004).

FSH biosynthesis is also regulated at transcriptional level by gonadal steroids, glucocorticoids and GnRH, and these actions are modulated by activin signaling. For example, androgen and activin synergistically stimulate FSHβ transcription, and the stimulation by androgen or activin alone is dependent on the presence of an intact Smad binding element (SBE) or androgen response element (ARE) (respectively) within the FSHβ promoter (Spady *et al.* 2004; Thackray & Mellon 2008). Glucocorticoids and activin also synergistically activate FSHβ transcription and this synergy occurs at the level of the FSHβ promoter (McGillivray *et al.* 2007). The role of GnRH in regulation of activin induced FSHβ transcription is more complex, because chronic administration (20–24 h) inhibits activin action (Shafiee-Kermani *et al.* 2007), whereas acute (4–6 h) GnRH synergistically enhances it (Gregory *et al.* 2004) and the interaction can also be species specific, making interpretation of heterologous reporter experiments challenging (Wang *et al.* 2008b; Lamba *et al.* 2008).

Activin also modulates transcription of LHβ subunit and GnRH receptor mRNA (Coss *et al.* 2005; Cherrington *et al.* 2005) as well as a number of other genes in LβT2 cells including *inhbb*, *inha*, *gdf9* and the 17β-HSD gene (Zhang *et al.* 2006). Activin also regulates the number of GnRH receptors and hence, response to GnRH (Gregory & Kaiser 2004). Thus, in addition to direct regulation of FSHβ biosynthesis, activin has important actions in modulating LHβ biosynthesis and release through regulation of GnRH action, together supporting a major role for activin in modulating neuroendocrine reproductive control.

2. Activin and glucose metabolism

Roles for activins and related members of the TGFβ family in regulating pancreatic β-cell number and/or function have been proposed for some time, and more recent results have refined those concepts, although the precise ligands and their actions in the pancreas remain to be definitively demonstrated. For example, activin can enhance glucose stimulated insulin secretion in β-cells from rats and humans in culture (Florio *et al.* 2000; Totsuka *et al.* 1988; Verspohl *et al.* 1993; Furukawa *et al.* 1995), and β-cell proliferation is enhanced with activin treatment (Li *et al.* 2004; Brun *et al.* 2004). In addition, all components of the activin/TGFβ signaling pathway have been identified in β-cells, including the ligands themselves, receptors, second messengers, and antagonists (Ogawa *et al.* 1993; Wada *et al.* 1996; Yamaoka *et al.* 1998). However, from these studies alone it was not possible to determine if activin or related ligands had physiological roles regulating β-cell proliferation or function *in vivo*.

Yamaoka et al (Yamaoka *et al.* 1998) demonstrated that transgenic overexpression of a dominant negative type II activin receptor in islets resulted in islet hypoplasia, reduced insulin content and impaired glucose tolerance, although the ratios of α- and β-cells was not altered. Although this study implicated activin in regulating β-cell proliferation, maturation, and possibly function, it was not possible in this model to differentiate between developmental effects and regulation ofβ-cells in adults. To circumvent this limitation, Smart et al (Smart *et al.* 2006) used a conditional transgenic strategy to overexpress Smad7 in β-cells. Smad7 is known as an inhibitory Smad which, unlike the signaling Smads, inhibits signaling in the activin/TGFβ pathway (Bhushan *et al.* 1998). Expression of Smad7 was inhibited in embryonic and neonatal mice by treating with doxycycline (Dox) and then induced in adult mice by removal of Dox, resulting in inhibition of the Smad2/Smad3 signaling pathway of activin and TGFβ (Smart *et al.* 2006). In these mice, β-cell gene expression, insulin content, serum insulin and glucose tolerance were all reduced compared to controls, and these effects were reversible with Dox administration (Smart *et al.* 2006). Thus, activin and/or related ligands have important roles in regulating β-cell number and function in rodents.

Further support for a role for activin in regulating β-cell proliferation and function was reported by Mukherjee et al (Mukherjee *et al.* 2007) when they created a global FSTL3 null mouse. Among other metabolic alterations, islets were nearly twice as large and contained mostly β-cells, insulin levels were slightly increased, and both glucose tolerance and insulin sensitivity, as assessed by glucose and insulin tolerance tests respectively, were enhanced. These observations suggest that removal of a natural activin antagonist resulted in increased activin bioactivity and increased β-cell mass and function (Mukherjee *et al.* 2007).

Although *in vitro* biological studies usually find nearly identical actions of activin A and B (Welt *et al.* 2002), activin B was shown to also use Alk7 as an alternative type I receptor (Tsuchida *et al.* 2004), suggesting activins A and B might have distinct actions *in vivo*. This concept is supported by the different phenotypes observed with activin A and B knockout mice (Matzuk *et al.* 1995b). In addition, Bertolino et al (Bertolino *et al.* 2008) found that Alk7 (and presumably, its ligand activin B) is a negative regulator of β-cell function, a finding in opposition to the reported stimulatory actions of activin A described above. Alk7 KO mice developed progressive hyperinsulinemia, reduced insulin sensitivity and impaired glucose tolerance. It appears that activin B acts by reducing glucose-stimulated calcium influx (Bertolino *et al.* 2008). Moreover, activin B KO mice have hyperinsulinemia consistent with activin B negatively regulating insulin production and secretion in β-cells (Bertolino *et al.* 2008).

Experimental reduction of β-cell mass has been shown to induce proliferation of ductal tissues within remaining pancreas and differentiation of insulin-producing cells to compensate for lost islets in a process known as neogenesis (Bonner-Weir 1994). More recently, it was found that activin expression is increased in ductal tissues and co-localized with the cytokeratin maker for ductal cells after reduction of β-cell mass with streptozotocin or partial pancreatectomy (Zhang *et al.* 2002). Activin was also found to increase proliferation of rat ductal cells *in vitro*, as well as to induce their differentiation into insulin producing cells expressing PDX-1, Ngn-3, and other genetic markers of β-cells (Park *et al.* 2007). Glucose-stimulated insulin secretion was also enhanced in these cells, and when transplanted, were able to reverse streptozotocin-induced diabetes in rats (Park *et al.* 2007). These observations suggest that activin may also influence replication and differentiation of islet cell progenitors located in ductal epithelium.

While activin's role in regulating β-cell proliferation and function appears to be important for maintaining normal glucose metabolism in adults, its role with other islet cell types remains relatively unexplored. However, activin was shown to decrease glucagon gene expression in

αcell lines and in human islets in culture and inhibit proliferation of α-cell lines in culture through inhibiting synthesis of Arx (Μαμιν & Πηιλιππε2006). These results suggest that activin A may have opposite roles in regulating α- and β-cells in adults in ways that enhance glucose uptake and decrease its synthesis, actions favorable to relieving symptoms of diabetes.

3. Activin and Stem Cell Differentiation

Embryonic stem cells, both mouse and human, were first cultured on mouse feeder cell layers to maintain pluripotency during expansion. The need for completely defined culture conditions for use in human embryonic stem cell (hESC) therapies drove efforts to identify culture medium components that facilitated hESC proliferation while maintaining pluripotency. Activin and other TGFβ family members signaling through Smads 2 and 3 were found to maintain hESC pluripotency and self-renewal (Beattie *et al.* 2005; James *et al.* 2005; Vallier *et al.* 2005) and that blocking activin signaling resulted in hESC differentiation (Vallier *et al.* 2005; Wu *et al.* 2008). Moreover, activin often interacts with other growth factors like fibroblast growth factor (FGF) to maintain pluripotency (Vallier *et al.* 2005; Xiao *et al.* 2006). The mechanism for these activities appears to involve inhibition of bone morphogenetic protein (BMP) signaling by the interaction of activin and FGF pathways (Willems & Leyns 2008), thereby sustaining expression of such stem cell genes as *Nanog, Oct4 and Sox2* through Smad binding to the Nanog promoter (Xu *et al.* 2008). It was additionally reported that this activin and nodal mechanism seems to be universal among mammals since it maintains pluripotency of epiblast stem cells from mice and rats, demonstrating that previously observed differences between mouse and human ESCs could be overcome and that both could be maintained in the pluripotent state with activin or nodal treatment (Ogawa *et al.* 2007; Brons *et al.* 2007).

Upon removal or inhibition of activin signaling, hESC differentiation ensues, either in embryoid bodies or as monolayers. Another goal of ESC research is to direct hESC differentiation into distinct pathways. Recapitulating normal embryonic development where activin is important in endoderm/mesoderm formation and patterning (Kumar *et al.* 2003; Tremblay *et al.* 2000), reapplication of activin induces hESCs to form endoderm (Yao *et al.* 2006b; Sumi *et al.* 2008; Funa *et al.* 2008) and even differentiate into distinct cell types such as insulin producing β-cells (Jiang *et al.* 2007; Shi *et al.* 2005; Jafary *et al.* 2008; Phillips *et al.* 2007; Frandsen *et al.* 2007), hepatocytes (Hay *et al.* 2008), parathyroid-like tissue (Bingham *et al.* 2008), neuroectoderm (Smith *et al.* 2008), blood cells (Nostro *et al.* 2008; Pearson *et al.* 2008), and cardiomyocytes (Kitamura *et al.* 2007), perhaps through modulation of other important developmental signaling systems such as sonic hedgehog (Mfopou & Bouwens 2008; Mfopou *et al.* 2007). Thus, activin has critical activities in regulating hESC pluripotency, and then differentiation into endoderm and its tissue derivatives that appears to be useful for directly ESC differentiation *in vitro*.

4. Activin and Immune Response

The importance of TGF-β family members in immune system function was first demonstrated by the targeted disruption of TGFβ1 in mice, which resulted in a severe multifocal inflammation (Shull *et al.* 1992). Subsequent studies revealed that activins (reviewed in (Jones *et al.* 2004b)) and BMPs (Gould *et al.* 2002; Lee *et al.* 2003; Hagen *et al.* 2007) are also important regulators of inflammatory responses in various cell types and organs.

Activin expression has been detected in many immune cells including monocytes (Eramaa *et al.* 1992; Abe *et al.* 2002), macrophages (Ebert *et al.* 2007), dendritic cells (Robson *et al.* 2008), T and B lymphocytes (Ogawa *et al.* 2008; Ogawa *et al.* 2006), and mast cells (Funaba *et al.* 2003; Cho *et al.* 2003), and its expression increases when these cells are activated by immune stimuli and toll-like receptor agonists. For example, microglial cells (quiescent CNS resident macrophage-like cells) and peritoneal macrophages released activin A and its binding

protein FST after treatment with TLR2 agonist Pam3Cys, TLR4 agonist LPS, or TLR9 ligand CpG (Cytosin-guanosin oligodesoxynucleotide 1668) (Ebert *et al.* 2007; Michel *et al.* 2003b). Blood dendritic cells and monocyte-derived dendritic cells express activin mRNA, and secretion of activin A protein was dramatically increased in the presence of *E coli*, or CD40 ligand (CD40L), or various TLR ligands (Robson *et al.* 2008). Secreted activin A then negatively regulated production of cytokines (IL-6, IL-12p70, TNF-α and IL-10) and chemokines (IL-8, IP- 10 RANTES, and MCP-1) induced by the CD40L/CD40 pathway in an autocrine/paracrine manner (Robson *et al.* 2008). These inhibitory functions of activin released by dendritic cells may play an important role in preventing uncontrolled release of cytokine/chemokine and recruitment of immune effectors within the local microenvironment (Robson *et al.* 2008). Activin was also expressed in activated $CD4^{+}CD25*^{minus;}$ T cells in the spleen and promoted differentiation of macrophages into alternatively (M-2 phenotype) activated macrophages (Ogawa *et al.* 2006). In addition, activin treatment inhibited IFN-γ- or LPS-induced NO_2 production, and suppressed LPS-induced pinocytosis and expression of TLR4 in mouse macrophages (Ogawa *et al.* 2006; Zhang *et al.* 2005; Wang *et al.* 2008a). Through direct action on resting B cells, activin A enhanced IgG and IgE production (Ogawa *et al.* 2008). These results indicate that activin is produced by a variety of cell types in the immune system and has regulatory actions on many of them, suggesting an intricate autocrine/paracrine system whereby activin modulates certain immune responses.

In addition to upregulation of activin expression in activated immune cells, activin expression is also stimulated by inflammatory mediators including LPS in endothelial cells (Wilson *et al.* 2006) and Sertoli cells (Okuma *et al.* 2005; Riccioli *et al.* 2006; Jones *et al.* 2007). Furthermore, increased activin A expression was also found in many other cell types in inflamed or wounded tissues (Hubner *et al.* 1996; Ota *et al.* 2003; Fumagalli *et al.* 2007; Kaitu'u-Lino *et al.* 2008). Thus, activin appears to play an important role both in immune response and immune reactions during wound repair, as well as perhaps influencing wound repair directly (Hubner *et al.* 1999). As a result of these immune system actions, elevated systemic activin A concentrations were found in the serum of patients with septicemia or angina (Michel *et al.* 2003a; Smith *et al.* 2004). In fact, a rapid increase in circulating activin A, peaking within 1 hour, was observed after LPS administration to sheep or mice, a response which preceded the systemic release of TNF-α, IL-6, IL-1β, and FST (Jones *et al.* 2007; Jones *et al.* 2004a). Blockade of activin action with FST treatment altered the profiles of TNF-α, IL-6, and IL-1β after LPS stimulation and enhanced survival from a lethal LPS dose, suggesting that activin modulates the release of other key proinflammatory cytokines (Jones *et al.* 2007). These results demonstrate that activin plays a crucial role in the inflammatory response and that blocking its actions may have therapeutic potential in treatment modalities for acute inflammatory disorders (Jones *et al.* 2007).

C. Summary

Over the past 5 years, substantial progress was made in elucidating the biological actions of activin in adult animals, taking advantage of novel techniques that allow regulation of the timing of genetic alterations in mice as models for human disease. The important actions so far enumerated indicate that activin has many important homeostatic functions in adult humans and is thus likely also to contribute to disease in those individuals where activin expression or bioactivity is altered. While no associations with human disease have yet been identified for activins A or B in genome wide association studies, FST and/or FSTL3 levels are altered, for example, in heart disease (Lara-Pezzi *et al.* 2008), suggesting that such associations might be uncovered in the near future as more individuals are genotyped. It is therefore likely that new actions of activin, as well as its involvement in disease, will be identified with additional research and that pharmaceuticals based on modulation of activin signaling having therapeutic value may be discovered in the next few years.

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