Activin A Signaling Induces Smad2, but Not Smad3, Requiring Protein Kinase A Activity in Granulosa Cells from the Avian Ovary*

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Ovarian granulosa cells are epithelial cells of endodermal origin (1). Granulosa cells are also termed follicle cells and, being the somatic cells closest to the germ cell, are thought to play important roles in oocyte development. In birds, granulosa cells form a monolayer of closely packed cuboidal cells embedded between the perivitelline layer, which surrounds the oocyte and constitutes the avian homologue of the mammalian zona pellucida, and a basal lamina, which separates granulosa cells from the theca layers (2). In vertebrates, ovarian granulosa cells are a main source of activins and inhibins (3), which belong to the TGF-β† superfamily of growth factors. They are formed by homo- and heterodimerization of three subunits: inhibin α, inhibin βA, and inhibin βB, yielding activin A (βAβA), activin B (βBβB), activin AB (βAβB), inhibin A (α2βA), and inhibin B (α2βB) (4). Like all TGF-β family members, activins signal via transmembrane receptor serine/threonine kinases of the TGF-β receptor family. Based upon structural and functional properties, the family consists of upstream type II receptors and downstream type I receptors. After the assembly of the ligand-induced heteromeric receptor complex, type II receptors transphosphorylate type I receptors, which in turn phosphorylate two intracellular signal transducers, the R-Smads and the receptor complex. Phosphorylation of SARA-presented R-Smads by type I receptors leads to dissociation of R-Smads and SARA from the receptor complex (6). Phosphorylated R-Smads are then able to recruit a common mediator Smad, the co-Smad Smad4, followed by the translocation of an R-Smad-co-Smad complex to the nucleus, which directly participates in the regulation of gene transcription (5, 7–9).

Importantly, despite acting downstream of the same ligands, Smad2 and Smad3 target largely distinct, yet overlapping sub-populations of TGF-β/activin-responsive genes (10). The inhibitory Smads (or I-Smads), Smad6 and Smad7, oppose the action of signal-transducing R-Smads by forming stable associations with activated type I receptors, thereby preventing R-Smad phosphorylation and activation (11).

A main task of ovary-derived activins and inhibins in the adult vertebrate is the regulation of availability of follicle-stimulating hormone (FSH), a key endocrine regulator of ovarian function (12–15). FSH is a glycoprotein secreted from the anterior pituitary gland, acting exclusively on ovarian granulosa cells and testicular Sertoli cells (16). These appear to be the only cell types expressing the corresponding FSH receptor, a typical seven-transmembrane domain G protein-coupled receptor. Ligand binding activates protein kinase A (PKA) through the elevation of intracellular cAMP. PKA in turn regulates transcription mainly via phosphorylation of the cAMP-responsive element binding protein (CREB)/ATF family CREB/ATF (17). Notably, FSH does not act solely via cAMP, since it also stimulates mitogen-activated protein kinase pathways via an alternatively spliced FSH receptor in porcine granulosa cells (18). Furthermore, FSH can activate mitogen-activated protein kinase pathways downstream of PKA in mammalian granulosa cells (19) as well as Sertoli cells.
(20, 21). There is increasing evidence suggesting that activins are not only endocrine regulators of FSH availability but also potent effectors of autocrine or paracrine intraovarian signaling. Activin receptors and downstream Smad proteins are present in rat ovaries (22–26), and activins apparently play important roles in regulation of folliculogenesis and follicle function (27–30). However, the knowledge about granulosa cells and how they respond to Smad signaling and its regulation is still rudimentary. Smad signaling is regulated at several different pathway levels by phosphorylation (31–33), protein degradation (34), and induction of I-Smad expression (35). However, most of these studies utilized cell lines overexpressing Smads at unphysiological levels. Hence, apart from I-Smad induction, little is known about the expression levels of the endogenous proteins, and even less is known about possible regulatory mechanisms that could control endogenous Smad expression in response to external stimuli.

We have previously established a novel culture system for chicken granulosa cells (cGC), which allows for propagation of functionally differentiated, primary avian granulosa cells (36). This system closely mimics the in vivo situation and greatly facilitates studies on endogenous Smad expression and regulation. Here we demonstrate a high abundance of Smad2 in this highly specialized epithelial cell type. We provide evidence that activin A signaling cooperates with PKA. Most interestingly, we show that Smad2, but not Smad3, expression is tightly regulated in response to FSH/activin A. We suggest that FSH/activin A-dependent Smad2 up-regulation constitutes a mechanism enabling FSH to change the transcriptional target read-out of activin signaling.

EXPERIMENTAL PROCEDURES

Animals, cGC Isolation, and Cell Culture—Brown Derco laying hens were obtained from Heindl Co. (Vienna, Austria) and maintained on layer’s mesh with water and feed provided ad libitum during a 12 h light of 16 h. All animal experimentation was in compliance with the regulations of the animal ethics committee of the University of Vienna. Hens were sacrificed by decapitation, and granulosa sheets were isolated from large preovulatory follicles as described previously (37) using minor modifications (36). Dispersed cells were resuspended and plated in Dulbecco’s modified Eagle’s medium (catalog no. 41965; Invitrogen) supplemented with 5% (v/v) fetal calf serum (Invitrogen), 50 U/ml penicillin, 50 µg/ml streptomycin, 50 µg/ml (1.4 µM) human pituitary FSH (Calbiochem), and 25 ng/ml (1.8 nM) human recombinant activin A (R & D Systems). cGC were cultivated under standard conditions (37 °C, 5% (v/v) CO₂) and, after reaching confluence, further propagated by splitting 1:2.

Growth Factors and Inhibitors—In general, cells were propagated in Dulbecco’s modified Eagle’s medium supplemented as described above. Before the start of an experiment, cells were shifted into supplemented Dulbecco’s modified Eagle’s medium, lacking FSH and activin A, and incubated for 24 h. Where applicable, inhibitors were added 30 min prior to growth factor supplementation. Inhibitor concentrations used were 10 µM PD98059, 3 µM KT5720, 3 µM H89 (Calbiochem), and 100 µM for 8-Br-cAMP (Sigma). Growth factors were added at 25 ng/ml (Amersham Biosciences). Northern blots were also exposed to x-ray films (Eastman Kodak Co.) to visualize RNA bands by autoradiography. All experiments were repeated at least three times. Northern blots and immunoblots show results of representative experiments.

RESULTS

Smad2 Expression and Localization in Vivo—We have cloned a full-length cDNA encoding chicken Smad2 (EMBL/GenBank™ accession number G3A18063). The encoded protein is 99% identical to human Smad2, readily suggesting a functional conservation of avian and human Smad2. We then analyzed the tissue-specific expression pattern by Northern blotting. Ovarian granulosa cells from large preovulatory follicles exhibited by far the highest Smad2 mRNA levels when compared with all other chicken tissues examined (Fig. 1A). Except for testes, which had a second, smaller transcript, only a single Smad2 mRNA species of 3.8 kb was detected. Further, immunofluorescence and immunoblotting experiments revealed that Smad2 protein was also highly expressed in cGC,

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whereas Smad2 expression in adjacent theca cells was undetectable (Fig. 1B). These results indicate a special role for Smad2 signaling in granulosa cells. Interestingly, Smad2 was not only highly expressed but also localized predominantly to the nucleus in freshly isolated cGC-sheets (Fig. 1C), indicating an active Smad2 signaling pathway. These results suggest a pivotal role of TGF-β-like signals for granulosa cell function in vivo.

**Activin A Activates the Smad Pathway and Induces Smad2 Expression**—Activin A represents a likely candidate to trigger nuclear accumulation and activation of Smad2 as seen in cGC freshly isolated from intact follicles. Hence, cultured cGC were treated with activin A, and Smad2 phosphorylation and nuclear translocation were examined. As an immediate response to activin A, Smad2 was modified by phosphorylation within 2 h, as shown by immunoblotting using phosphospecific antibodies recognizing phosphorylated P-Smad2 (Fig. 2A). Immunostaining of cGC with anti-Smad2 antibodies revealed efficient translocation of the protein into the nucleus within the same time period (Fig. 2B). If cGC were cultured in the absence of activin A, Smad2 expression was rapidly down-regulated. To test whether loss of Smad2 expression was due to a lack of stimulating ligand, we added activin A to depleted cells and followed Smad2 by Northern analysis (Fig. 3). Indeed, the addition of activin A reinduced and thus increased Smad2 mRNA levels. However, simultaneous addition of activin A and FSH caused Smad2 induction in a synergistic way, leading to a massive up-regulation of Smad2 mRNA (Fig. 3). Although we cannot rule out that changes in mRNA stability also contribute to Smad2 regulation, the observed regulatory effects are most likely due to transcriptional control. Interestingly enough, the induction was specific for Smad2, since expression levels of the functionally and structurally closely related Smad3 mRNA remained completely unaffected by treatment with activin A or activin A/FSH (Fig. 3).

**Smad2 Induction by Activin A Requires Active PKA**—Smad2 protein showed the same regulation pattern as Smad2 mRNA. Whereas FSH alone failed to induce Smad2, a combination with activin A boosted Smad2 induction, giving rise to very high Smad2 protein levels (Fig. 4A). These data indicate functional cooperation between activin A and FSH signaling. Because FSH action could not be blocked by the MEK inhibitor
PD98059, FSH-triggered MEK/ERK activation cannot explain the observed effects (Fig. 4A). Further, the cAMP analogue 8-Br-cAMP efficiently simulated the presence of FSH regarding Smad2 induction (Fig. 4B), demonstrating that FSH acts through cAMP. Notably, in the absence of activin A, neither FSH nor 8-Br-cAMP elevated Smad2 expression (Fig. 4, A and B). These results indicate that cGC require activin A in vitro to sustain in vivo levels of Smad2. Moreover, the involvement of FSH-triggered, cooperating PKA signaling seemed likely. Again, Smad2 mRNA showed the same regulation pattern as the corresponding protein, indicating transcriptional regulation (data not shown).

To further analyze the role of the FSH pathway, two PKA inhibitors, KT5720 and H89, were tested for their ability to block FSH- and 8-Br-cAMP-induced effects on activin A-triggered Smad2 induction. As shown in Fig. 5, both PKA inhibitors abrogated Smad2 induction by activin A alone was completely abolished by both PKA inhibitors, indicating that activin A signaling requires basal PKA activity to induce Smad2 (Fig. 5A). The protein levels of the cadherin and β-catenin controls remained unchanged during these treatments. Further, Northern blotting showed that Smad2 mRNA induction was significantly attenuated by the PKA inhibitors KT5720 (Fig. 5B) and H89 (data not shown), fully confirming the protein data. By sharp contrast, the steady state levels of the mRNA encoding the structurally and functionally related Smad3 remained constant under all tested conditions (Fig. 5B). Taken together, these results clearly demonstrate a requirement for both activin A and PKA activity to induce Smad2. Neither activin A nor PKA activity alone were sufficient, implying that activin A and FSH

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**Fig. 3.** Activin A specifically induces Smad2 but not Smad3. Cultured cGC were treated for 24 h with indicated growth factor combinations, followed by total RNA isolation and Northern blot analysis with cDNA probes specific for chicken Smad2 and Smad3, respectively. Methylene blue staining of rRNA served as loading control.

**Fig. 4.** FSH boosts activin A-dependent Smad2 induction and acts via cAMP. A, cultured cGC were treated with activin A and FSH alone or in combination of both in the absence or the presence of 10 μM PD98059 for 24 h. Protein extracts were analyzed by Western blotting with anti-Smad2 antibodies. B, FSH was replaced by 8-Br-cAMP to mimic the effect of FSH. ZO-1 served as loading control.

**Fig. 5.** PKA inhibitors block Smad2 induction. A, cells were treated with the indicated growth factors and inhibitors. After 24 h, protein extracts were subjected to immunoblotting using Smad2 antibodies. As loading controls, blots were incubated with anti-pancadherin or anti-β-catenin antibodies, both of which remain unregulated under the conditions used. B, cells were treated as above, and total RNA was prepared after 24 h and subjected to Northern blotting using cDNA probes specific for Smad2 and Smad3, respectively.
trigger converging signaling pathways, leading to enhanced Smad2 expression in cGC.

**Smad2 mRNA Induction Requires Activin A-dependent de novo Protein Synthesis**—In order to learn more about the mechanism of cooperation between activin A and the cAMP pathway, Smad2 induction was studied in time course experiments. Smad2 induction is a delayed process, since Smad2 mRNA became elevated only after about 6 h following the addition of growth factors. More importantly, the induction is completely abolished by cycloheximide, strongly suggesting that at least one of the pathways inducing Smad2 requires de novo protein synthesis (Fig. 6A). To further characterize this putative protein synthesis step, cGC were pretreated with activin A for 24 h followed by 8-Br-cAMP addition and Northern analysis. Pretreatment was carried out in the presence of PKA inhibitors to block basal PKA activity and to avoid subsequent Smad2 induction during the preincubation period. Smad2 expression remained constantly low during the preincubation. However, mRNA levels were induced as early as 2 h after 8-Br-cAMP addition (Fig. 6B). After 4 h, protein levels were also elevated (Fig. 6C). Hence, in activin A-pretreated cells, PKA activation was swift, inducing Smad2 without any delay. By contrast, 8-Br-cAMP pretreatment followed by activin A application completely failed to induce Smad2, even after 24 h of activin A treatment (Fig. 6, B and C). Thus, the suspected de novo protein synthesis step is triggered by activin A rather than 8-Br-cAMP.

This notion was further supported by an additional experiment (Fig. 6D). Cells were pretreated with activin A/H89 in the presence of cycloheximide. Whereas cells pretreated in the absence of cycloheximide showed the expected rapid Smad2 mRNA induction upon 8-Br-cAMP stimulation (Fig. 6D, lanes 4 and 5), cells pretreated in the presence of cycloheximide were not responsive. Smad2 mRNA levels in these cells remained constant during 8-Br-cAMP stimulation (Fig. 6D, lanes 6–8). We conclude that the presence of cycloheximide during the preincubation blocked activin A-triggered protein synthesis, which seems to be a crucial prerequisite for subsequent Smad2 induction by 8-Br-cAMP. Taken together, activin A is required to sustain high Smad2 expression in cultured cGC. Smad2 induction by activin A is PKA-dependent in vitro, since it is boosted by PKA activators but abrogated by PKA inhibitors in combination with FSH, activin A restored high Smad2 levels as observed in the in vivo situation, indicating that the same growth factor combination might be active in signaling within the intact follicle. Interestingly, Smad3 expression remained completely unregulated by these growth factors. These results suggest that FSH alters the readout of activin signaling by specifically promoting Smad2-dependent transcriptional regulation but repressing Smad3-dependent transcriptional regulation in avian granulosa cells.

**DISCUSSION**

Granulosa cell differentiation is necessary for proper follicular development and ovarian function. Hence, understanding signal transduction events driving these processes is pivotal for understanding ovarian biology. A key regulator of granulosa cell function is FSH, whose availability is tightly regulated by TGF-β-like growth factors such as the activins and inhibins produced within the ovary. Recent evidence suggests that activins also play important roles in intraovarian signaling (27–30). The activin signals are transduced by cytoplasmic Smad proteins, leading to transcriptional activation or repression of target genes, yet the knowledge about expression and regulation of these proteins within ovarian tissues is still scarce, and many models describing Smad signaling used artificial overexpression systems. However, increasing Smad gene dosage frequently causes ligand-independent induction of TGF-β-responsive promoters (38, 39), emphasizing the requirement for studies in primary, untransfected cells. In this work, we show that the R-Smad Smad2 is expressed at the highest levels in cGC among all chicken tissues examined. The nuclear localiza-
tion of Smad2 readily suggests a functional signaling pathway in vivo. Notably, high Smad2 expression in cultured cells requires a functional interplay between activin A and FSH/PKA signaling. Our results suggest that cultured cGC may respond to a similar growth factor combination in vivo, stressing the importance of activin A as a major intraovarian regulator in birds.

Smad2 Is Highly Expressed and Functionally Active in cGC in Vivo—Smad2 mRNA and protein levels in cGC by far exceed levels present in most other chicken tissues, as shown by Northern blots and immunofluorescence experiments. Notably, Smad2 protein levels are relatively low in follicular theca cells, whereas mRNA levels seem rather high (Fig. 1, A and B). Given the extraordinary high expression in cGC and the close proximity of both cell types in the follicle, this could be explained by a contamination of cGC mRNA preparations with thecal mRNA. Smad2 not only is present in large quantities, but is also functionally active, since the protein shows predominant nuclear localization in freshly isolated cGC. Thus, active TGF-β-like ligands must exist within the follicle to drive nuclear accumulation of Smad2. Notably, granulosa cells actively secrete activin A, and they express cognate activin receptors (22, 23, 26), suggesting autocrine signaling in ovarian cells. Activin is therefore a possible candidate for an autocrine ligand that triggers activation and nuclear translocation of Smad2. Although we cannot rule out other activins or TGF-βs as possible stimuli, we show that Smad2 is phosphorylated and rapidly translocated into the nucleus in response to activin A in cultured cGC. This is also essential to maintain the epithelial phenotype as well as differentiated functions of cGC in vitro (36).

Activin A cooperates with FSH in different aspects of cGC function, including a synergistic activation of genes associated with granulosa cell differentiation, including the perivitelline membrane protein chZPC and inhibin α (36). Likewise, activin A and FSH also induce proliferation-associated proteins such as cyclin D2 and proliferating cell nuclear antigen in rat granulosa cells, thereby stimulating granulosa cell proliferation (40). Hence, both granulosa cell differentiation and proliferation appear to be regulated, at least in part, by functional cross-talk of these signaling pathways that converge at the level of transcription. Our data identify activin A as a major autocrine effector and intracellular Smad2 as a specific intracellular target at the convergence of at least two signaling pathways.

FSH Cooperates with Activin A through PKA Activation—Our results suggest a mechanism of cooperation between FSH and activin A signal transduction. In general, FSH mainly signals via cAMP-dependent PKA activation, although it also activates the MEK/ERK cascade. This occurs either in parallel via a differentially spliced receptor (18) or downstream of cAMP (20). MEK/ERK-dependent FSH effects are readily abrogated by the specific MEK inhibitor PD98059 in porcine granulosa cells (18). Concerning Smad2 induction in cGC, a role of the MEK/ERK cascade in FSH signaling can be excluded, because Smad2 induction by activin A/FSH still occurs in the presence of high doses of PD98059. FSH can be substituted by 8-Br-cAMP, and Smad2 induction is blocked by PKA-specific inhibition. Hence, FSH-induced PKA activation is crucial for Smad2 induction in the presence of activin A. Activin A alone is sufficient for a moderate Smad2 induction, which is still abolished by PKA inhibitors, suggesting that activin A depends upon basal PKA activity. A rapid PKA activation by activin A, as demonstrated in zebrafish embryos (41), cannot account for avian Smad2 induction, since PKA activation by FSH or 8-Br-cAMP is not sufficient to trigger a response. In zebrafish embryos, PKA is involved in the activin-dependent induction of early mesodermal genes (41). However, to the best of our knowledge, our data provide the first direct evidence for a mutual dependence of activin A and PKA activity during signaling events in cells from adult vertebrates.

Smad2 mRNA induction by activin A/FSH is completely abrogated by cycloheximide, supporting the idea that protein synthesis is required for Smad2 mRNA induction. Short stimulation with 8-Br-cAMP is sufficient to trigger a swift response in activin pretreated cells. However, the presence of cycloheximide during preincubation with activin A abrogates Smad2 induction during subsequent 8-Br-cAMP stimulation. These results suggest that protein synthesis is triggered by activin A rather than PKA. The nature of the elusive protein(s) is unclear at present. Based on our data, we propose a model in which activin A exerts indirect effects by promoting the synthesis of an as yet unknown protein, whose function requires PKA activity to stimulate Smad2 transcription. PKA stimulates transcription through at least two mechanisms; one is the well-established phosphorylation and subsequent activation of CREB/ATF transcription factors, which bind to CREs in cAMP-responsive promoters (17). A second mechanism operates in granulosa cells, namely the FSH-induced histone H3 phosphorylation through PKA, which facilitates transcription of select gene products (42). Both mechanisms could also operate in the avian ovary. CREB/ATF binding requires presence of a CRE motif in target gene promoters. Genes responsive to a synergistic activation by activin A/FSH including inhibin α (36), proliferating cell nuclear antigen (40), and chZPCβ with CRE indeed contain CREs (17). The only known Smad2 promoter sequence is the human Smad2 promoter (43). However, based on extensive analysis, it lacks a CRE motif. Because synergy of activin A/FSH also exists in mammalian granulosa cells (40), our results hint at a general importance of this cooperation. However, the exact molecular mechanisms remain to be elucidated.

Selective Smad2 Induction May Affect Overall Smad Signaling Specificity—Smad2 and Smad3 are structurally and functionally related, sharing an ability to transduce extracellular signals. However, whereas Smad3 has DNA binding activity, Smad2 has not due to a 30-amino acid insertion in the MH1 domain adjacent to the DNA binding region of Smad3 (44, 45). Thus, the existence of both transducers might indicate a regulatory function or imply a differential set of target genes. This regulatory function could be further enhanced by selective recruitment of positively or negatively acting co-regulators. The knowledge of TGF-β/activin target genes and their precise cellular roles is rather scarce, and studies dealing with Smad DNA binding activity and Smad-activated transcription rely on a very limited amount of reporter constructs. Nevertheless, a number of studies found distinct specificities in transcriptional activation mediated by Smad2 and Smad3 (38, 39, 46–48) as well as differences in co-factor recruitment (49). Whereas phenotypic differences between Smad2−/− and Smad3−/− knock-out mice (50) also arise from differential spatial and temporal expression during embryonic development, studies in Smad2−/− and Smad3−/− mouse embryonic fibroblasts revealed two distinct, partially overlapping sets of target genes, strongly supporting the notion of pronounced functional differences between Smad2 and Smad3 (10).

Both Smad2 and Smad3 bind to the cognate type I receptors mediated by SARA (6), and they appear to exhibit similar binding affinities (51). Hence, the phospho-Smad2/phospho-Smad3 ratio should closely resemble the Smad2/Smad3 ratio. A

* N. Bausek, personal communication.
selective induction of Smad2 as presented in this paper will therefore shift the equilibrium from Smad3-Smad4 complexes toward Smad2-Smad4 complexes. Whereas a direct comparison and quantification of expression levels of different proteins is very difficult to achieve, our results clearly show that only Smad2 levels, and not those of Smad3, are highly variable in response to activin A/FSH. Smad2 induction depends upon the presence of activin A and PKA activity. Consequently, activin A signaling in the presence of PKA activity will preferentially target Smad2-responsive genes, whereas in the absence of PKA activators, activin A signaling will predominantly target Smad3-responsive genes. To support this hypothesis, reporter assays with constructs selectively responding to either Smad3 or Smad2 should be a feasible approach. Despite technical difficulties in the transfection of primary, epithelial cGC, our efforts toward the delivery of reporter constructs into cultured cGC are continuing. In summary, our results suggest an important role for FSH in altering the readout of Smad signaling in response to activin A in cGC, modulating the relative activities of two distinct signaling branches downstream of the same cell surface effector.

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