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# Autocrine regulation of Leydig cell differentiated functions by insulin-like growth factor I and transforming growth factor beta ☆

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**Abstract**

The expression and the maintenance of specific differentiated function of Leydig cells are regulated not only by gonadotropin but by locally produced factors, which may act as autocrine regulators. Many factors, in particular growth factors, have been postulated to have such a type of effect on testicular cells, but very few fulfilled the three criteria required to establish a paracrine/autocrine role: (a) presence of receptors and biological action on local cells; (b) local secretion regulated by physiological signals; and (c) blockade of the factor or its receptors must modify the function of local cells. In the present work we demonstrate that two factors, insulin-like growth factor 1 (IGF-I) and transforming growth factor β1 (TGFβ1) fulfilled the three criteria: (a) IGF-I stimulates the transcription of the genes encoding Leydig cell differentiated function, leading to an enhanced steroidogenic responsiveness to LH/hCG; (b) Leydig cells (LC) express and secrete IGF-I and this secretion is enhanced by hCG; and (c) incubation of LC with IgG anti-IGF-I, but not with IgG-control, markedly reduced the steroidogenic responsiveness to LH/hCG. In contrast to IGF-I, TGFβ is a potent inhibitor of LC differentiated function. Moreover, LC express TGFβ1 mRNA and secrete this peptide. To prove that the locally produced TGFβ has an autocrine role, LC were transfected with 10 μM of an antisense oligonucleotide (AON) complementary to the translation initiation region of TGFβ1 mRNA. Transfection with AON but not with sense deoxynucleotide induces a complete disappearance of TGFβ immunoreactivity in LC and an enhanced hCG-induced testosterone production by LC. This increased steroidogenic responsiveness was associated with a significant enhancement of both LH/hCG receptor mRNA and P450c17 mRNA. Taken together, the above results show that both factors play an autocrine role, although opposite, on Leydig cell function. 

## 1. Introduction

The two main testicular functions are androgen production and spermatogenesis which take place in two different compartments. Steroidogenesis occurs in the vascularized interstitial or intertubular compartments composed of Leydig cells, macrophages, blood vessels and lymphatics and the sheath of myoid or peritubular cells that surrounds the seminiferous tubules. Spermatogenesis occurs within the avascular seminiferous tubule, which is made up exclusively of Sertoli cells and germ cells. The two compartments are separated by the blood–testis barrier which consists of three filters: the surrounding layer of myoid cells, the basement membrane of the tubule and the last and more restricted filter, the Sertoli–Sertoli cell tight junctions, which are established at the time of puberty. This barrier restricts the passage of many factors into the abluminal compartment and lumen of the seminiferous tubule. Thus, all the nutrients, hormones and growth factors required for the very energy-demanding process of spermatogenesis, must be supplied by the ultrafiltrate of interstitial fluid, containing some of the secretory products of Sertoli cells.

Normal function of the testis has long been recognized to be dependent on the pituitary-synthesized gonadotropins. Notwithstanding these requirements

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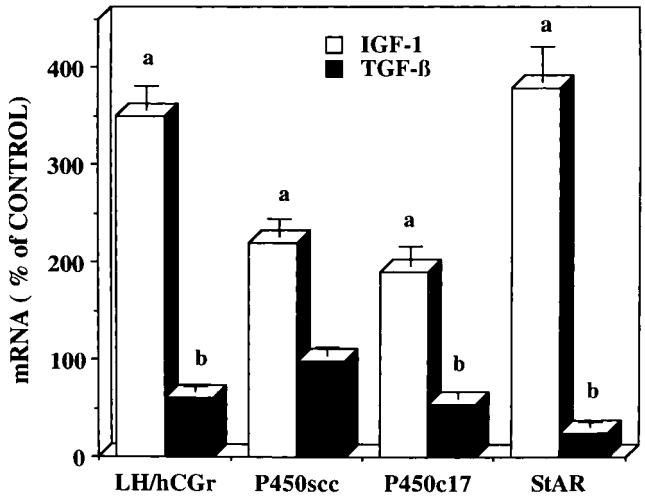


Fig. 1. Effects of IGF-I and TGFβ on LH/hCG receptor, P450scc, P450c17 and 3β-HSD mRNA in porcine Leydig cells. Cells were cultured without or with IGF-I (50 ng/ml) or TGFβ (2 ng/ml) for 2 days, and the mRNA levels determined by Northern blot. A letter on a bar indicates a significant difference from the control.

for gonadotropins, numerous reports over the past several years have clearly indicated that locally produced factors may play an important role in the regulation of Leydig cell functions. Recent articles have extensively reviewed this aspect [1–6]. Thus, many factors have been shown to be produced locally and/or to be able to have pleiotropic effects on Leydig cells. This has led to the hypothesis that these factors may play a paracrine/autocrine role on Leydig cell differentiation and function. However, to establish that any factor has such a role, three criteria must be fulfilled: (1) presence of receptors and biological action on local cells; (2) local secretion regulated by physiological signal; and (3) blockade of the factor or its receptor by antibody, antagonist or antisense oligodeoxynucleotides, must modify the function of local cells.

This paper will concentrate on two growth factors, insulin-like growth factor I (IGF-I) and transforming growth factor β1 (TGFβ1) and demonstrate that in pig Leydig cells these two factors fulfil the three criteria described above and, therefore, that this local production could play an autocrine role in the differentiation and function of Leydig cells.

## 2. Leydig cells are the site of receptors and action of IGF-I

IGF type I receptor has been demonstrated in Leydig cells of several species by binding studies, cross-linking and immunohistochemistry [7–9]. Moreover, in both pig [8] and rat [9,10] Leydig cells, LH/hCG up-regulates these receptors. In the rat and before puberty, IGF-I stimulates the proliferation of precursor and immature Leydig cells, and the differentiation of immature into mature Leydig cells [11,12]. Although the steroidogenic action of IGF-I alone, if any, is very small, pretreatment with IGF-I stimulates hCG-supported cAMP and testosterone production by cultured Leydig cells of murine [13,14] and porcine [8,15] origin. The response to cAMP analogs was also enhanced, suggesting that IGF-I may potentiate LH/hCG action at sites both proximal and distal to cAMP generation. Further studies have demonstrated that IGF-I increased LH/hCG receptor number and mRNA, the activity and the mRNA levels of several steroidogenic enzymes [8,16] and StAR (Steroidogenic Acute Regulatory protein) (Fig. 1). The stimulatory effect on these mRNA was mainly at the transcriptional level [16].

Further evidence of the positive role of IGF-I on Leydig cell differentiation and function was obtained by in vivo studies. First, in human, isolated GH deficiency [17] or GH resistance, as in the case of Laron syndrome [18], are associated with micropenis, suggesting a decreased fetal Leydig function during the second half of pregnancy, delayed puberty and poor response to exogenous hCG [17] which, in the case of GH deficiency, is very often improved following treatment with GH [17,19]. Second, administration of GH but also of IGF-I to Snell dwarf mice during 7 days increases the number of testicular LH/hCG receptor and the steroidogenic response to hCG [20]. Third, the strongest evidence that IGF-I is crucial in the development and function of Leydig cells came from studies of IGF-I gene knockout mice [21]. In these animals, the testes were reduced in size more than expected from the degree of dwarfism, the number and the volume of Leydig cells were markedly reduced, as well as plasma testosterone levels and the in vitro basal and LH-stimulated testosterone production by testicular slices were impaired.

## 3. Leydig cells are the site of IGF-I production

The expression of IGF-I mRNA has been demonstrated in Leydig cells of several species [21–26]. The levels of IGF-I mRNA in Leydig cells were reduced in hypophysectomized rats but they increased following GH administration [22,24]. These results, however, are contradictory with the lack of effect of human GH on dwarf mouse testicular IGF-I mRNA and on dwarf rat testicular IGF-I peptide [27,28]. Similarly, contradictory results have been reported concerning the role of LH/hCG in testicular IGF-I. In vivo, administration of hCG increases IGF-I mRNA levels on rat Leydig cells [23,24], whereas in vitro, the opposite effects have been reported [22]. In vitro, IGF-I peptide is secreted by rat and porcine Sertoli cells [29,30] as well as by

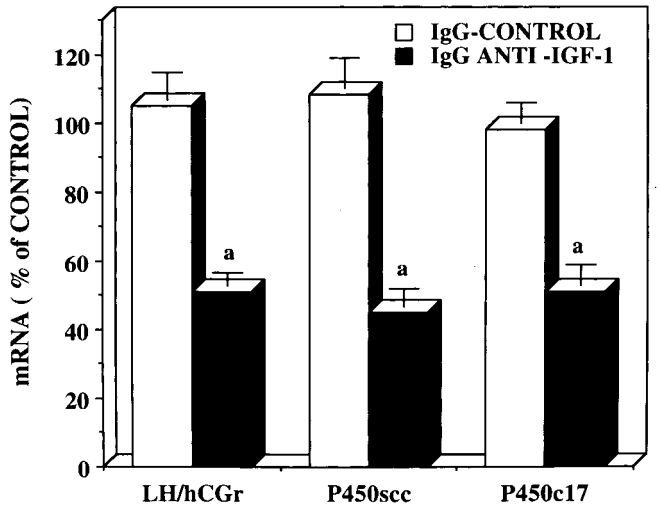


Fig. 2. Effects of IGF-I antiserum on porcine Leydig cells. Cells were treated with 50 μg of IgG prepared from non-immune serum or IGF-I antiserum for 2 days. At the end of this period, the mRNA levels of LH/hCG receptor, P450scc and P450c17 were evaluated by Northern blot. The results are percent of cells cultured in the same medium without IgG. A letter indicates a significant difference from the control.

Leydig cells [31,32] of the same species, and this secretion was stimulated in a dose-dependent fashion by hCG and FSH, respectively [32].

## 4. Inhibition of secreted IGF-I by antibodies inhibits Leydig cell functions

Although endogenous secreted IGF-I plays a positive trophic effect on Leydig cell functions, inhibition of its action by specific antibodies should produce the opposite effects. Treatment of rat Leydig cells with IGF-I antiserum decreased the steroidogenic responsiveness to LH, when compared to cells treated with non-immune serum [33]. Moreover, the IGF-I antiserum almost completely blocked the positive action of Sertoli cells on Leydig cell steroidogenic capacity. Similarly, treatment of porcine Leydig cells with increasing concentrations of IgG prepared from IGF-I antiserum decreased the steroidogenic responsiveness to hCG stimulation, whereas the response of cells treated with the same amounts of IgG prepared from non-immune serum had the opposite effect [34]. This decrease in the steroidogenic responsiveness of Leydig cells treated with IgG anti-IGF-I was associated with a decrease of LH/hCG receptor, P450scc and P450c17 mRNA levels (Fig. 2). Taking together the in vivo and in vitro results, it appears that IGF-I is one of the factors for which there is convincing evidence to postulate that, in addition to its endocrine role, this factor plays a paracrine/autocrine role in the regulation of Leydig cells.

## 5. Leydig cells are the site of reception and action of TGFβ

Porcine Leydig cells contain specific TGFβ receptors of high affinity [35] and the developmental testicular expression of mRNAs for the three types of receptor has been analyzed in the rat [36]. TGFβ has pleiotrophic effects on Leydig cells. Treatment with TGFβ reduced hCG-induced testosterone production in pig [37,38], rat [39,40] and mouse [41] Leydig cells. In both pig [37] and rat [39] Leydig cells, TGFβ reduced also the steroidogenic response to cAMP derivatives, suggesting an impairment of the steroidogenic pathway. Further studies have shown that in pig Leydig cells TGFβ causes a decrease of LH/hCG receptor number and mRNA as well as of P450c17 and StAR mRNA [16] (see also Fig. 1).

## 6. Leydig cells are the site of production of TGFβ

Several groups have demonstrated that rat, mouse and pig LC express TGFβs mRNAs [42–45] and TGFβ-like immunoreactive material [45–49]. In the rat [43] and the mouse testis [44], TGFβ1 and TGFβ3 have been shown to be expressed in Sertoli cells and peritubular cells throughout testicular development. In immature pig testis, both Leydig cells and Sertoli cells express TGFβ1 mRNA and protein [45]. During fetal life, immunostaining for TGFβ1 appears in the rat primordial Sertoli cells on fetal day 14.5, increases until day 16.5, and becomes faint from fetal day 18.5 onward, whereas in fetal Leydig cells a positive reaction for TGFβ1 appeared on day 16.5 and became very intense during late fetal life and persisted until postnatal day 20 [48]. Contradictory results have been reported concerning the presence of immunoreactivity for TGFβ1 in adult rat Leydig cells, disappearance at the time of puberty [47], or positive but slight staining [48]. This discrepancy may result from differences in the antibodies used.

In vitro studies have shown that rat Sertoli cells and peritubular cells secrete the three TGFβ isoforms [43], whereas pig Sertoli cells and Leydig cells secrete mainly TGFβ1 [45]. In cultured Sertoli cells from 20-day-old rats, FSH reduces TGFβ2 mRNA and protein [43], whereas in pig Sertoli cells FSH decreases TGFβ1 mRNA and protein [45]. In contrast, in rat fetal testis, FSH stimulates TGFβ1 protein secretion, but not its mRNA levels and these effects were potentiated by LH [46].

Taken together, the above results clearly demonstrate that testicular somatic cells express and secrete TGFβs, but there is some diversity in their regulation according to the isoform, the species and/or the devel-

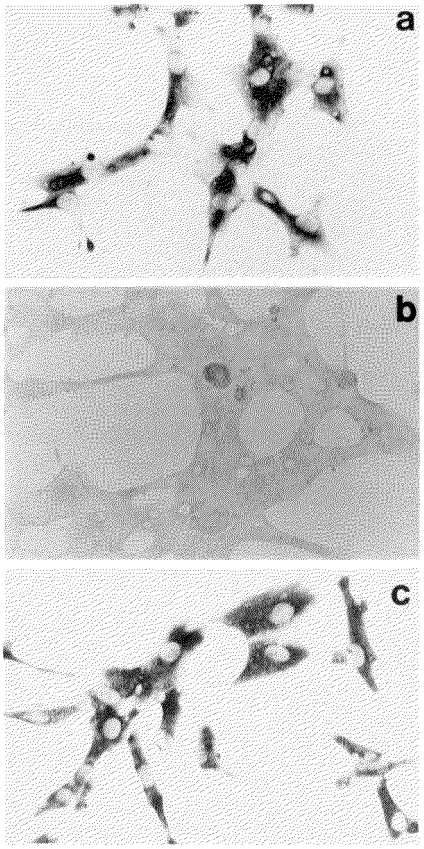


Fig. 3. TGFβ1 protein content in control (a) AON (b) and SON (c) treated Leydig cells. The immunocytochemical staining was performed using a specific TGFβ1 antibody.

opment stage. However, Leydig cells express and secrete predominantly TGFβ1.

## 7. Inhibition of TGFβ1 synthesis in Leydig cells increases their steroidogenic activity

This study has used two approaches to block the effects of endogenous secreted TGFβ1 in Leydig cells: TGFβ1 antibodies and oligonucleotide antisense. The first approach did not give clear-cut results probably because the TGFβ1 antibody used recognizes only the active form of this peptide [45] but Leydig cells, as many other cells, secrete the peptide in a latent form [45]. For the second approach, this study used antisense (AON) and sense (SON) unmodified 15-bases deoxyribonucleotides, corresponding to the translation initiation region of TGFβ1 mRNA. Cells were transfected for 24 h by a cationic liposome-mediated transfection method, as described before [50] and cultured for 44 h.

Neither AON nor SON modified TGFβ mRNA levels (data not shown). In contrast, AON, but not SON, completely blocked TGFβ1 protein synthesis as revealed by immunocytochemistry (Fig. 3).

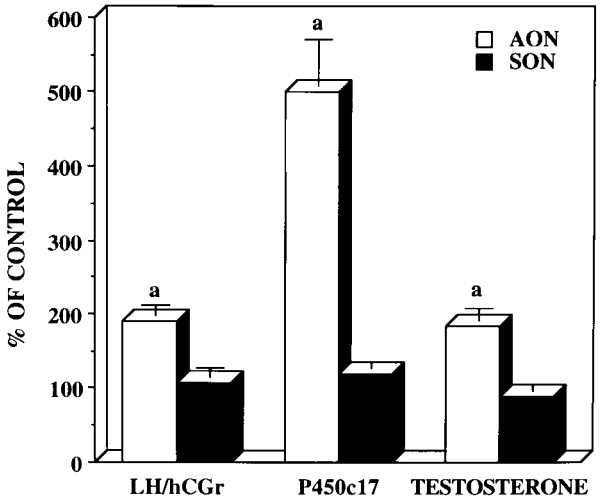


Fig. 4. Effects of antisense (AON) and sense (SON) on LH/hCG receptor mRNA, P450c17 mRNA and hCG-induced testosterone production. Cells were transfected for 24 h without or with 10 μM of AON or SON. After 44 h of culture, RNA was extracted and analyzed by Northern blot. Other cells were stimulated with hCG ($10^{-9}$ M) and the testosterone production measured after 2 h. A letter indicates a significant difference from control.

To investigate the biological consequences of TGFβ1 protein synthesis inhibition in Leydig cells, two types of parameters were measured. First, this study evaluated the mRNA levels of LH/hCG receptor and P450c17, since, as indicated above (Fig. 1), exogenous TGFβ1 reduced both. The results of Fig. 4 clearly show that TGFβ1 AON, but not SON, increased LH/hCG receptor and P450c17 mRNA levels by about 2- and 5-fold (Fig. 4). Second, the steroidogenic responsiveness to hCG was studied and it was demonstrated that AON, but not SON, increased by about 1.6-fold the hCG-induced testosterone production (Fig. 4).

All these data show that the inhibition of TGFβ1 synthesis by AON produces effects which are opposite to that induced by exogenous TGFβ1 in Leydig cells.

## 8. Conclusions

Leydig cells from several species contain specific

Table 1
Effects of TGFβ and IGF-I on Leydig cells differentiated functions

| | TGFβ1 | IGF-I |
|---|---|---|
| hCG R mRNA | ↓ | ↑ |
| StAR mRNA | ↓ | ↑ |
| P450scc mRNA | → | ↑ |
| P450 17α mRNA | ↓ | ↑ |
| hCG-induced testosterone production | ↓ | ↑ |

receptors from both IGF-I and TGFβ and these peptides are able to regulate, but in opposite manner (Table 1), the expression of several specific Leydig cell functions. In addition, Leydig cells express and secrete both IGF-I and TGFβ and their secretion is regulated by physiological signals. Finally, the inhibition of action or synthesis of these peptides caused the opposite effects to those produced by the addition of these factors. In addition to these autocrine effects, it is likely that these factors also play a paracrine role, since both are also secreted by the other somatic testicular cells. In vivo studies in human and experimental animals have confirmed the crucial role of IGF-I on Leydig cell differentiation and function, but such studies are still required to confirm the physiological relevance of TGFβs in the regulation of Leydig cell function.

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