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## **Fxr $\alpha$ gene is a target gene of hCG signaling pathway and represses hCG induced steroidogenesis.**

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**Running title:** FXR $\alpha$  & LH.

**Key words:** Leydig, testis, hCG signaling, FXR $\alpha$ , steroidogenesis.

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### **Highlights.**

- \* hCG signaling pathway regulates *Fxr $\alpha$*  expression
- \* cAMP signaling pathway regulates *Fxr $\alpha$*  expression
- \* FXR $\alpha$  agonist exposure leads to a negative feedback of the hCG-induced steroid synthesis.

## **Abstract.**

**The bile acid receptor Farnesoid-X-Receptor alpha (FXR $\alpha$ ), a member of the nuclear receptor superfamily, is well known for its roles in the enterohepatic tract. In addition, FXR $\alpha$  regulates testicular physiology through the control of both endocrine and exocrine functions. The endocrine function of the Leydig cells is mainly controlled by the hypothalamo-pituitary axis via LH/chorionic gonadotropin (CG). If FXR $\alpha$  was demonstrated to control the expression of the *Lhcgr* gene, encoding the LH receptor; the impact of the LH/CG signaling on the *Fxr $\alpha$*  expression has not been defined so far. Here, we demonstrate that hCG increases the *Fxr $\alpha$*  gene expression through the protein kinase-A signaling pathway. *Fxr $\alpha$*  is then involved in a negative feedback of steroid synthesis. These data improve our knowledge of the local control of the testicular steroidogenesis with the identification of the link between the hypothalamo-pituitary axis and the FXR $\alpha$  signaling pathway.**

## **1. Introduction.**

In the last years, it has been demonstrated that the nuclear receptor Farnesolid-X-Receptor- $\alpha$  (FXR $\alpha$ ; NR1H4) is expressed in testis from birth to adulthood<sup>1</sup>. In early postnatal age, it has been described that FXR $\alpha$  controls the establishment and the maintenance of the mouse spermatogonial stem cell pool<sup>2</sup>. It was also described that FXR $\alpha$  is a regulator of the endocrine function of the testis in pubertal and adult male mice<sup>3,4</sup>. Indeed, it has been demonstrated that the activation of the FXR $\alpha$  signaling pathways in Leydig cells, by administration of the specific synthetic agonist GW4064, represses testosterone synthesis<sup>5</sup>. This repression of steroidogenesis results from the induction of the expression of two nuclear receptors *Shp* (*Small heterodimer partner; NR0B2*) and *Dax-1* (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene-1; NR0B1), two repressors of steroidogenesis<sup>6</sup>. This critical role of FXR $\alpha$  signaling pathway on the control of the testicular endocrine function is supported by the observed phenotype induced by the exposure to bile acids (BA) at pubertal period, a critical developmental window relying on testosterone levels<sup>6</sup>. Indeed, pubertal bile acid exposure of male mice leads to altered fertility associated with default in germ cell differentiation, which is correlated with an increased rate of spermatocytes apoptosis. This later is associated with a default in testosterone synthesis as demonstrated by the rescue of the phenotype by exogenous testosterone administration<sup>6</sup>. Recently, several studies highlight links between steroid homeostasis and FXR $\alpha$ , which was sustained by the combined impacts of FXR $\alpha$  antagonist (namely stigmasterol) and estrogenic

endocrine disrupters (such as bisphenol-A) on testicular physiology<sup>7</sup>.

The control of the Leydig cell functions, including steroidogenesis, is mainly mediated by the hypothalamo-pituitary-gonadal axis *via* LH/chorionic gonadotropin (CG)<sup>8</sup>. Interestingly, it has been demonstrated that part of the action of FXR $\alpha$  is through the control of the expression of the *Lhcgr* gene, encoding the LH receptor<sup>6</sup>. Steroidogenesis is a complex process where cholesterol serves as the initial substrate. The conversion of cholesterol to pregnenolone takes place within mitochondria. The transport of cholesterol to the mitochondria is ensured by the steroidogenic acute regulatory protein (StAR)<sup>9</sup> or peripheral benzodiazepine receptor (translocator protein, TSPO)<sup>10,11</sup>. Then, multiple enzymatic steps involving the cytochrome P450-11A1 (Cytochrome P450, Family 11, Subfamily A, Polypeptide 1, CYP11A1)<sup>11</sup>, the 3 $\beta$ -hydroxy-steroid dehydrogenase (3 $\beta$ HSD)<sup>12</sup> and the cytochrome P450-17A1 (Cytochrome P450, Family 17, Subfamily A, Polypeptide 1, CYP17A1) lead to the production of testosterone<sup>13</sup>.

The present study is focused on the analysis of how *Fxr* $\alpha$  expression is regulated by the hypothalamo-pituitary (HP) axis and more particularly by hCG signaling pathway within the Leydig cells. *In vivo* treatment with human chorionic gonadotropin (hCG) results in the increase of testicular *Fxr* $\alpha$  mRNA accumulation. *In vitro* approaches, using the mouse Leydig MA10 cell line, demonstrate the involvement of protein kinase A (PKA) signaling pathways. This highlights the fact that *Fxr* $\alpha$  is a critical regulator of Leydig homeostasis and thus of the testicular endocrine function.

## **2. Materials and Methods**

### **2.1. Animals**

C57BL/6J (Charles River, France) mice were housed in controlled temperature rooms with 12-hour light, 12-hour dark cycles and had ad libitum access to food and water.

Mice were injected intraperitoneally with hCG (5 IU, equivalent to 1.42mM; Sigma-Aldrich) diluted in NaCl 0.09%.

This study was conducted in accordance with current regulations and standards approved by Institut National de la Santé et de la Recherche Médicale Animal Care Committee.

### **2.2. Endocrine investigations**

Progesterone was measured in the medium of MA10 cell culture, and plasma and intratesticular testosterone was measured as previously described using a commercial kit (ADI-900-011 & ADI-900-065- Enzo Life Sciences) was used for the assays<sup>14</sup>.

### **2.3. Real-time RT-PCR**

RNAs from testis samples were isolated using TRIzol (Invitrogen Corp). cDNA was synthesized from total RNA with the SuperScript II First-Strand Synthesis System (Life Technologies) and random hexamer primers. Real-time PCR measurement of individual

cDNAs was performed using SYBR green dye to measure duplex DNA formation with the Eppendorf Realplex system. Sequences of the primers were given in previous studies: *Actin*, *Fxr $\alpha$* , *Star*, *Hsd3b1*, *Shp* and *Dax-1*<sup>2,14,15</sup>. Standard curves were generated with pools of testis cDNA from animals with different treatments. Results were analyzed using the delta-delta-cycle threshold values method.

**Interstitial cell enrichment.** For the generation of data on enriched interstitial cells compared to tubular preparations or whole testis samples, the methodology used relies on mechanical and enzymatic processes for dissociating the fractions. The interstitial enriched cells and tubular compartments were generated as described in previous study<sup>2</sup>. Briefly, testes from 90-day-old male mice were decapsulated and incubated for 20 min at 33 °C in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 (1:1), transferrin (5 µg/ml), insulin (4 µg/ml) and vitamin E (0.2 µg/ml) medium containing collagenase (0.8 mg/ml) (Life Technologies, Invitrogen, Cergy-Pontoise, France). Extracts were collected by centrifugation for 10 min at 200 g and the pellet was resuspended in fresh medium. Following two successive sedimentations, the supernatant containing the interstitial cells was centrifuged and the pellet was suspended in fresh medium at a final concentration of 107 cells/ml.

The enrichment was established through the analysis of specific markers. The mRNA expression pattern was characterized by low mRNA accumulations of Fsh receptor (*fshr* : a *Sertoli cell marker*), and of *Plzf* or *Stra8* (markers of germ cell lineage) ; and high expression of Leydig gene such as Lh receptor (*lhcg*) in the interstitial cells ; whereas tubular compartment showed an opposite pattern, supporting the interstitial enrichment of the samples (**Figure Supplemental 1C**).

## 2.4 Cell culture experiments

MA10 cells were previously used<sup>14</sup> and were maintained at 37°C in an atmosphere of 5% CO<sub>2</sub> with Waymouth (Life Technologies) containing 100-U/mL penicillin and 100-µg/mL streptomycin supplemented with 10% horse serum. On day 0, MA10 cells were seeded at 400 × 10<sup>3</sup> cells per well in 6-well plates and allowed to adhere overnight MA10 cells were transfected with small interfering RNA (siRNA) using Jet Prime (Ozyme, Saint Quentin Yvelines, France) in six-well plates (400,000 cells per well). The siRNA directed against *Fxr $\alpha$* <sup>2</sup>, as well as control siRNA (siGfp)<sup>16</sup>, was transfected at 5 ng per well. When 48 hours after the transfection had passed, cells were washed twice with 1 × PBS, and medium without serum was applied with various ligands or vehicles. For hCG experiments, vehicle was NaCl 0.09%, for forskolin (Fsk) (Sigma-Aldrich), 8-BromocAMP (8BrcAMP) (Sigma-Aldrich), protein kinase A inhibitor H89 (H89) (Sigma-Aldrich), flutamide (Sigma-Aldrich), R1881 (Sigma-Aldrich), Casodex or vehicle was dimethylsulfoxide (1:1000; Sigma-Aldrich).

Time after the hCG exposures, cells were washed twice with 1× PBS, and medium without serum was applied with (DMSO, 1/1,000) or GW4064. Then, cells were harvested 4 hours later, and mRNA extractions were performed.

### 3. Results and Discussion

#### 3.1. hCG increases *Fxrα* expression in vivo.

To better define *in vivo* the potential links between FXR $\alpha$  and the hCG signaling pathways, we first analyzed the impact of hCG treatment on *Fxrα* mRNA expression. Twelve hours of treatment with 5 IU of hCG resulted in a significant increase in *Fxrα* mRNA accumulation in 3 months old adult mouse testis (**Figure 1A**) Moreover, we show that the increase of the *Fxrα* mRNA accumulation by hCG exposure was also observed at pubertal age (20-days old male mice), a critical developmental window regarding the establishment of the Leydig cell homeostasis (**Supplemental Figure 1A**). Note that at both ages, as expected, *Star* mRNA accumulation (**Figure 1A & Supplemental Figure 1A**) and both intra-testicular and plasma testosterone levels (**Figure 1B & Supplemental Figure 1B**) were increased following treatment with hCG.

In addition as expected, both plasma and intra-testicular testosterone were higher in adult mice than in pubertal animals. In that line the respective mean of plasma levels were of 20ng/ml in adult and 1ng/ml in pubertal mice. Regarding intra-testicular testosterone mean of concentrations were of 3000ng/mg in adult and 24ng/mg in pubertal mice.

If FXR $\alpha$  signaling alters Leydig, Sertoli and germ cell physiologies<sup>2,14,15</sup>, in adult mice FXR $\alpha$  is mainly expressed in Leydig cells and to a lesser extent in spermatogonia<sup>2,6</sup>. To ensure that hCG signaling alters the *Fxrα* mRNA accumulation within Leydig cells, we have performed the analysis of the *Fxrα* mRNA accumulation in enriched interstitial and tubular populations isolated from vehicle or hCG exposed adult male mice. As expected<sup>5</sup>, data confirmed that *Fxrα* mRNA is mainly accumulated in interstitial compartment (**Figure 1C**), where its expression is significantly up-regulated by the hCG signaling pathway.

If previous studies<sup>6</sup> have identified the impact of FXR $\alpha$  signaling on the expression of *Lhcgr* gene, the present data support the hypothesis of a crosstalk in the other way round.

#### 3.2. hCG increases *Fxrα* expression in Leydig cells.

To confirm the above-mentioned data of the regulation of the *Fxrα* expression by hCG, the main regulator of Leydig cell function, we used the well-characterized mouse MA10 Leydig cell line. *Fxrα* mRNA accumulation was increased after hCG exposure since the dose of 0.25ng (**Figure 2A**). Interestingly, the progesterone levels were increased since this same dose of 0.25 ng (**Figure 2B**). The effect of hCG on *Fxrα* accumulation was observed since 2

hours and up to 24 hours after treatment (**Figure 2C**). As expected, *Star* mRNA accumulation was positively regulated by hCG (**Figure 2A & 2C**). Interestingly, the progesterone levels were increased since 2 hours and then significantly decreased significantly after 12 hours (**Figure 2D**).

### **3.3. *Fxr*α regulation by hCG involves cAMP-PKA signaling pathway.**

In order to decipher the downstream signaling pathways induced by the LH/CG receptor, we used the adenylate cyclase activator, Forskolin (Fsk) in *in vitro* experiment using MA10 Leydig cell line through dose and time experimental approaches. Results show that Fsk induced the *Fxr*α mRNA accumulation at 2.5μM since 2 hours of treatment (**Figure 3 A and B**). As expected, *Star* mRNA accumulation was increased after exposure to Fsk (**Figure 3 A and B**). As expected the progesterone level was increased since the lowest dose used. The increased was seen after 2 hours of treatment and then slightly decreased at 8 hours after exposure (**Figure 3C**).

The involvement of cAMP pathway was confirmed using 8BrcAMP (**Figure 3D**). Treatment for 4 hours with 8BrcAMP resulted in induction of *Fxr*α and of *Star* mRNA accumulations (**Figure 3D**). In Leydig cells, PKA pathway plays an important role in the control of steroidogenesis. To define whether PKA was involved in *Fxr*α regulation by cAMP, we used a pharmacological inhibitor of PKA, namely H89. Pretreatment with H89 abolished the effect of hCG on *Fxr*α mRNA accumulation (**Figure 3E**). As expected the treatment with H89 limits the impact of hCG on progesterone synthesis (**Figure 3F**). If previous data<sup>14</sup> suggest that the FXRα signaling pathway might be a regulator of the hCG stimulated testicular steroidogenesis; the present data validate the interaction in the opposite way. This highlights new molecular mechanisms of the interactions between FXRα signaling pathway and the hypothalamo-pituitary axis. This is of importance as the Leydig cell functions, including steroidogenesis, control the initiation of sexual maturation and germ cell survival at adulthood<sup>17</sup>.

### **3.4. hCG regulation of FXRα expression is not mediated through testosterone or progesterone.**

Consistently with the increase of progesterone levels since 2-hours after hCG exposure *in vitro* and the increase of testosterone *in vivo* following hCG administration, one might have think that the effect of hCG on *Fxr*α mRNA levels could be due to the progesterone or testosterone respectively synthesized in MA-10 cells or *in* Leydig cells (*in vivo*). To answer this question, we performed experiments using progesterone or testosterone exposures (**Figure 4**). Data showed that in MA10 cell line, the expression of

*Fxrα* was not regulated following administration of progesterone or testosterone (**Figure 4A & 4B**). These data were confirmed using the androgen receptor (AR) synthetic agonist R1881. Indeed, no impact on *Fxrα* mRNA accumulation was observed following 2 or 4 hours of R1881 administration (**Figure 4C**). In addition, the inhibition of AR through the use of AR antagonist, namely flutamide, did not impact the *Fxrα* expression *in vitro* (**Figure 4D**).

We next performed *in vivo* experiments using exposure to testosterone or flutamide. If testosterone led to a decrease of *Star* mRNA accumulation, no effect was observed on *Fxrα* expression (**Figure 4E**). Consistent with the lack of impact of androgen pathway on *Fxrα* expression, no effect of flutamide was observed on *Star* or *Fxrα* expression (**Figure 4F**).

To decipher more precisely the potential links between the hCG-induced steroid production and the following regulation of the *Fxrα* expression; we next have tested the potential impact of the inhibition of the androgen pathway on the response to hCG both *in vitro* (MA10 cells) and *in vivo*. Data showed that *in vitro* inhibition of the androgen receptor by flutamide did not interact with the impact of hCG on *Fxrα* expression (**Figure 5A**). These data were consistent with those obtained for *Star* mRNA accumulation and progesterone levels (**Figure 5A & 5B**). These observations were confirmed using Casodex another AR antagonist (**Figure 5C & 5D**).

However, surprising data showed that *in vivo*, flutamide counteracted in part the effect of hCG on *Star* and *Fxrα* mRNA accumulations (**Figure 5E**). These data were supported by the results obtained for intra-testicular testosterone levels (**Figure 5F**). These data suggest that the impacts of flutamide exposure through AR inhibition might somehow impact the hCG signaling pathway. This hypothesis was supported by the fact that flutamide led to a decrease of the mRNA accumulation of *Lhcgr* (**Figure 5G**). Interestingly, it could be explained that flutamide counteract the impact of hCG as both molecules synergistically impact the mRNA accumulation of the *Lhcgr* gene (**Figure 5G**).

### 3.5. hCG stimulation sensitizes MA10 cells to FXRα agonist exposure.

So far there is only few data supporting the role of the “backward” of hCG signaling on FXRα receptor. We have hypothesized that the activation of FXRα might participate to a negative control of the induction of hCG-induced steroidogenesis in order to avoid a sustained hyper-steroidogenesis. This is a possible hypothesis as steroid production will decrease at a certain time following hCG induction as seen *in vitro* in MA10 cells where the levels of progesterone will significantly decrease 12 hours after hCG exposure (**figure 2C**). To analyze such hypothesis, we have performed on MA-10 cells a kind of time dependent experiment that supports the idea of a feedback control. We first have analyzed the consequences of the exposure to FXRα agonist namely GW4064. This later has been



described to be an inhibitor of testicular steroidogenesis<sup>5</sup>. We have validated this in the MA10 Leydig cell line where the impact of 12-hours exposure to GW4064 was mediated by FXR $\alpha$  as supported by the fact that the use of a siRNA directed against *Fxr* $\alpha$  abolished the effect of GW4064 on *Star*, *Hsd3b1*, *Shp* and *Dax-1* mRNA accumulations (**Supplemental Figure 2**). To define the impact of hCG on FXR $\alpha$  activity, the MA10 cells were pre-treated with hCG for 6 or 12 hours in order to induce the FXR $\alpha$  expression and then cells were exposed to GW4064 for 2 or 4 hours. Data demonstrated that no combined effect of GW4064 and hCG was observed after 6 hours of hCG on *Star* and *Shp* mRNA accumulation (**Supplemental Figure 3**). In contrast, after 12 hours of hCG treatment exposure to GW4064 acted in a most potent manner than when administrated alone on many gene expressions (**Figure 6**). This might reflect the necessity to have a production of FXR $\alpha$  protein. Indeed data showed that the pre-treatment to hCG led to an enhanced response to GW4064 since 2 hours of treatment (**Figure 6A**). Indeed, after 2 or 4 hours of treatment with GW4064, the fold induction of *Shp* and *Dax-1* in the hCG-GW4064 treated cells were greater compared to GW4064. In that line, a significant decrease of *Star* and *Hsd3b1* mRNA was observed in the hCG/GW4064 treated group compared to GW4064 alone (**Figure 6A & 6B**). Consistent with the above results, following the 12-hours treatment of hCG, exposures to GW4064 (2 or 4 hours) hours led to a complete abrogation of the elevated progesterone levels compared to hCG treated cells returning to levels comparable to the vehicle treated cells (**Figure 6C**). **These data are of interest as it has been demonstrated that 12 hours after hCG exposure the level of steroids are decreasing (Figure 2C)**. This kinetic is of interest as it is consistent with the fact that cells will be sensitive to FXR $\alpha$  agonist in the same timeframe. Interestingly, these effects on the mRNA accumulations of *Star*, *Hsd3b1* and *Shp* as well as on steroid production were abolished by a siRNA directed against *Fxr* $\alpha$  (**Figure 7A, 7B, 7C & 7D**). Surprisingly, the cells transfected with the siRNA directed against *Fxr* $\alpha$  did not show increased of progesterone levels in response to hCG. Combined these data reinforce the links between FXR $\alpha$  signaling pathways and steroid metabolism<sup>18</sup>. We demonstrate that FXR $\alpha$  acts in a negative feedback of steroid synthesis following hCG exposure. This is crucial as endocrine homeostasis is a critical physiological process, as alteration could lead to various diseases.

#### 4. Conclusion.

The present data show that hCG through the PKA signaling pathway regulates the expression of the *Fxr* $\alpha$  gene. The effect is not due to the subsequent steroid production induced by LH as demonstrated by the experiments using testosterone, R1881, flutamide or casodex. **It has to be noticed that experiments were performed with low dose of hCG**

(0.25nM). In addition, experiments were performed in condition of serum starvation, to avoid interference of serum with BA signaling pathways, and measurement of steroid synthesis was performed at least 18 to 25 hours following hCG exposure, combined this might explain in part the low basal levels of progesterone and the slight increase following hCG exposure.

In conclusion, the present work confirms the links between FXR $\alpha$  and the efficiency of the testicular endocrine function. This point has been sustained in the last decades with the large impact of endocrine disrupters, on animal and human health<sup>19</sup>. It is interesting to note that critical collaborative links have been made between FXR $\alpha$  signaling pathways and exposure to estrogenic endocrine disrupters such as bisphenol-A<sup>7</sup>.

In conclusion, this work supports the idea of a complex crosstalk between FXR $\alpha$  and the LH/CG signaling pathways in order to strictly regulate the Leydig cell homeostasis and particularly the steroid synthesis. Our work allowed us to define molecular interactions. We previously showed<sup>14</sup> that LH signaling, through the regulation of the steroidogenic factor one (SF-1; NR5A1), the liver receptor homolog-1 (LRH-1; NR5A2) and CREB activities and in the meantime the lower expression of *Shp* and *Dax-1* resulting in the expected, stimulates steroidogenesis. Here our data demonstrate that LH/CG induces the expression of FXR $\alpha$  in a sustained time frame (from 2 to 24h hours). This impact sensitizes the Leydig cells to FXR $\alpha$  agonist, that counteract the increase of steroid synthesis due to hCG exposure. This thus conducts to a subsequent decrease of the synthesis of steroids. These data suggest that this regulation of FXR $\alpha$  by LH might reflect a negative feedback to regulate Leydig endocrine function.

So far, there is only few data supporting the role of the back-word of LH on FXR $\alpha$  receptor. The time course experiments performed on MA10 cells show that the initial effect of hCG is first seen on steroidogenic genes, and the regulation of the *Fxr $\alpha$*  mRNA accumulation is associated to the impact FXR $\alpha$  signaling 12 hours after hCG induction. These data support the idea of a feedback control to avoid a sustained hyper-steroidogenesis.

In the present paper mainly focused on the regulation of the *Fxr $\alpha$*  gene by LH signaling pathways within the Leydig cells. The data clearly highlight the crosstalk between both pathways. Even though it was not analyzed in the present paper, it has been demonstrated that in the context of some liver diseases, BA could have impact of the hypothalamus and thus on LH and FSH production by the pituitary. Thus further data using transgenic mouse models (*Fxr $\alpha$*  knock-out mice) will be needed to better define the complete crosstalk mechanisms at the hypothalamus/pituitary levels that could lead to an alteration of the hypothalamo-pituitary-gonadal axis and in turn the alteration of testicular physiology and

particularly steroid synthesis.

Combined with previous data on the links between the FXR $\alpha$  signaling and the estrogenic endocrine disrupters; the present data define FXR $\alpha$  as a critical actor to explore in more details in order to increase our knowledge of pathologies associated with altered testicular endocrine function such as fertility disorders.

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## Figure legends

### Figure-1: hCG increases *Fxrα* expression in testis.

**A**, Testicular mRNA accumulation of *Fxrα* and *Star* normalized to *Actin* mRNA levels in whole testes of adult C57BL/6J treated with vehicle or 5-IU hCG for 12 hours.

**B**, Intratesticular and plasma testosterone levels measured in adult C57BL/6J treated with vehicle or 5-IU hCG for 12 hours.

**C**, mRNA accumulation of *Fxrα* and *Star* normalized to *Actin* mRNA levels in testicular tubular and interstitial compartments of adult C57BL/6J treated with vehicle or 5-IU hCG for 12 hours.

In all panels, n = 12 per group and data are expressed as the mean ± SEM. Statistical analysis: \*,  $p < .05$ ; \*\*,  $P < .01$ .

### Figure-2: hCG increases *Fxrα* expression in Leydig cells.

**A**, mRNA expression of *Fxrα* and *Star* normalized to *36b4* levels in MA10 Leydig cell line exposed to vehicle or hCG (from 0.0025nM to 25nM) over 24 hours; n = 12 per group.

**B**, Relative progesterone levels in medium of MA10 cells exposed to vehicle or hCG (from 0.0025nM to 25nM) over 24 hours; n = 12 per group.

**C**, mRNA expression of *Fxrα* and *Star* normalized to *36b4* levels in MA10 Leydig cells exposed to vehicle or hCG (0.25nM) for 2, 4, 6, 10 or 24 hours.

**D**, Relative progesterone levels in medium of MA10 cells exposed to vehicle or hCG (0.25nM) for 2, 4, 6, 10 or 24 hours.

In all of the panels, n = 12 per group (3 to 4 Independent experiments) data are expressed as the mean ± SEM. Statistical analysis: \*,  $P < .05$ ; \*\*,  $P < .01$ . \*: compared to vehicle treated group; #: compared to 2hours hCG treated group.

### Figure-3: *Fxrα* regulation by hCG involves cAMP-PKA signaling pathway

**A**, mRNA expression of *Fxrα* and *Star* normalized to *36b4* levels in MA10 Leydig cells exposed to vehicle or Fsk (from 2.5μM to 10μM) over 4 hours.

**B**, mRNA expression of *Fxrα* and *Star* normalized to *36b4* levels in MA10 Leydig cell line exposed to vehicle or Fsk (5μM) for 2, 4, 6, or 8 hours.

**C**, Relative progesterone levels in medium of MA10 cells exposed to vehicle or Fsk (from 2.5μM to 10μM) over 4 hours or in medium of MA10 cells exposed to vehicle or Fsk (5μM) for 2, 4, 6 or 8 hours. \*: compared to vehicle treated group; #: compared to 6 hours Fsk treated group.

**D**, mRNA expression of *Fxrα* and *Star* normalized to *36b4* levels in MA10 Leydig cell line

exposed to vehicle or 8BrcAMP (50, 100 or 200  $\mu$ M) over 4 hours.

**E**, mRNA expression of *Fxr $\alpha$*  and *Star* normalized to *36b4* levels in MA10 Leydig cells pretreated with H89 for 2 hours and then treated with vehicle or hCG (0.25nM) for 4 hours.

**F**, Relative progesterone levels in medium of MA10 cells exposed pretreated with H89 for 2 hours and then treated with vehicle or hCG (0.25nM) for 4 hours.

In all panels, n = 12 per group and data are expressed as the mean  $\pm$  SEM. Statistical analysis: \*,  $p < .05$ ; \*\*,  $P < .01$ .

**Figure-4: *Fxr $\alpha$*  expression is not affected by testosterone or progesterone signaling.**

**A**, mRNA expression of *Fxr $\alpha$*  and *Star* normalized to *36b4* levels in MA10 Leydig cells exposed 2 or 4 hours to vehicle or progesterone (at  $10^{-6}$  or  $10^{-7}$ M).

**B**, mRNA expression of *Fxr $\alpha$*  and *Star* normalized to *36b4* levels in MA10 Leydig cells exposed 2 or 4 hours to vehicle or testosterone (at  $10^{-8}$  or  $10^{-9}$ M).

**C**, mRNA expression of *Fxr $\alpha$*  and *Star* normalized to *36b4* levels in MA10 Leydig cells exposed 2 or 4 hours to vehicle or R1881 (at  $10^{-6}$ M).

**D**, mRNA expression of *Fxr $\alpha$*  and *Star* normalized to *36b4* levels in MA10 Leydig cells exposed 2 or 4 hours to vehicle or flutamide (at  $10^{-7}$ M).

**E**, mRNA expression of *Fxr $\alpha$*  and *Star* normalized to *36b4* levels in MA10 Leydig cells exposed 2 or 4 hours to vehicle or Casodex (at  $10^{-8}$ M) over 4 hours.

**F**, mRNA expression of *Fxr $\alpha$*  and *Star* normalized to *36b4* levels in interstitial cells of testis of mice exposed for 12 hours to vehicle or testosterone (200 $\mu$ g/mice).

**G**, mRNA expression of *Fxr $\alpha$*  and *Star* normalized to *36b4* levels in interstitial cells of testis of mice exposed for 12 hours to vehicle or flutamide (40mg/kg/mice).

For experiments on MA10 cells, n = 12 per group and data are expressed as the mean  $\pm$  SEM. Statistical analysis: \*,  $p < .05$ ; \*\*,  $P < .01$ .

For experiments on mice, n = 5 to 6 per group and data are expressed as the mean  $\pm$  SEM. Statistical analysis: \*,  $p < .05$ ; \*\*,  $P < .01$ .

**Figure-5: hCG regulation of *Fxr $\alpha$*  expression is not mediated through androgen signaling in vitro.**

**A**, mRNA expression of *Fxr $\alpha$*  and *Star* normalized to *36b4* levels in MA10 Leydig cells exposed 12 hours with veh or hCG and then 4 hours to vehicle or flutamide.

**B**, Relative progesterone levels in medium of MA10 cells exposed 12hours with veh or hCG and then 4 hours to vehicle or flutamide.

**C**, mRNA expression of *Fxr $\alpha$*  and *Star* normalized to *36b4* levels in MA10 Leydig cells

exposed 12 hours with veh or hCG and then 4 hours to vehicle or Casodex.

**D**, Relative progesterone levels in medium of MA10 cells exposed 12h with veh or hCG and then 4 hours to vehicle or Casodex.

**E**, mRNA expression of *Fxrα* and *Star* normalized to *36b4* levels in interstitial cells of testis of mice exposed for 12 hours with vehicle, hCG, flutamide or hCG+flutamide.

**F**, Relative intratesticular levels of testosterone in mice exposed for 12 hours with vehicle, hCG, flutamide or hCG+flutamide.

**G**, mRNA expression of *Lhcgr* normalized to *36b4* levels in interstitial cells of testis of mice exposed for 12 hours with vehicle, hCG, flutamide or hCG+flutamide.

For experiments on MA10 cells, n = 12 per group and data are expressed as the mean ± SEM. Statistical analysis: \*,  $p < .05$ ; \*\*,  $P < .01$ .

For experiments on mice, n = 5 to 6 per group and data are expressed as the mean ± SEM. Statistical analysis: \*,  $p < .05$ ; \*\*,  $P < .01$ . \*: compared to vehicle treated group; #: compared to hCG treated group.

**Figure-6: hCG stimulation sensitizes MA10 cells to FXRα agonist exposure.**

**A**, mRNA expression of *Star*, *Hsd3b1*, *Shp* and *Dax-1* normalized to  $\beta$ -actin levels in MA10 cells pre-treated 12h with vehicle or hCG and then 2 hours with vehicle or GW4064.

**B**, mRNA expression of *Star*, *Hsd3b1*, *Shp* and *Dax-1* normalized to  $\beta$ -actin levels in MA10 cells pre-treated 12h with vehicle or hCG and then 4 hours with vehicle or GW4064.

**C**, Relative progesterone levels in medium of MA10 cells pre-treated 12h with vehicle or hCG and then 2 or 4 hours with vehicle or GW4064.

In all panels, n = 12 per group and data are expressed as the mean ± SEM. Statistical analysis: \* and #,  $p < .05$ . \*: compared to vehicle group; #: compared to hCG group.

**Figure-7: hCG stimulation sensitizes MA10 cells to FXRα agonist exposure.**

**A**, mRNA expression of *Star*, *Hsd3b1* and *Shp* normalized to  $\beta$ -actin levels in MA10 cells transfected with a siGfp or a siRNA directed against *Fxrα* pre-treated 12h with vehicle or hCG and then 2 hours with veh or GW4064.

**B**, mRNA expression of *Star*, *Hsd3b1* and *Shp* normalized to  $\beta$ -actin levels in MA10 cells transfected with a siGfp or a siRNA directed against *Fxrα* pre-treated 12h with vehicle or hCG and then 4 hours with veh or GW4064.

**C**, Relative progesterone levels in medium of MA10 cells transfected with a siGfp or a siRNA directed against *Fxrα* pre-treated 12h with vehicle or hCG and then 2 hours with veh or GW4064.

**D**, Relative progesterone levels in medium of MA10 cells transfected with a siGfp or a siRNA directed against *Fxr $\alpha$*  pre-treated 12h with vehicle or hCG and then 4 hours with veh or GW4064.

In all panels, n=12 from 3 independent experiments. Data are expressed as the means  $\pm$  SEM. Statistical analysis: #, \*, p<0.05; \*\*, p<0.01, \*\*\*, p<0.005 vs. respective control group.

\* compared to vehicle group; #: compared to hCG group.

### **Supplemental Figure 1**

**A**, Testicular mRNA accumulation of *Fxr $\alpha$*  and *Star* normalized to *Actin* mRNA levels in whole testes of pubertal C57BL/6J at 21-days treated with vehicle or 5-IU hCG for 12 hours.

**B**, Intratesticular and plasma testosterone levels measured in pubertal C57BL/6J treated with vehicle or 5-IU hCG for 12 hours. \*, p<0.05.

**C**. The mRNA expression pattern characterized by low mRNA accumulations of Fsh receptor (*fshr* : a Sertoli cell marker), and of Plzf or Stra8 (markers of germ cell lineage) ; and high expression of steroidogenic genes such as Lh receptor (*lhcg*) in the interstitial cells ; whereas tubular compartment showed an opposite pattern. measured in pubertal C57BL/6J treated with vehicle or 5-IU hCG for 12 hours. \*, p<0.05.

### **Supplemental Figure 2.**

mRNA expression of *Star*, *Hsd3b1*, *Shp* and *Dax-1* normalized to  $\beta$ -*actin* levels in MA10 cells transfected with a siGfp or a siRNA directed against *Fxr $\alpha$*  treated 12h with vehicle GW4064.

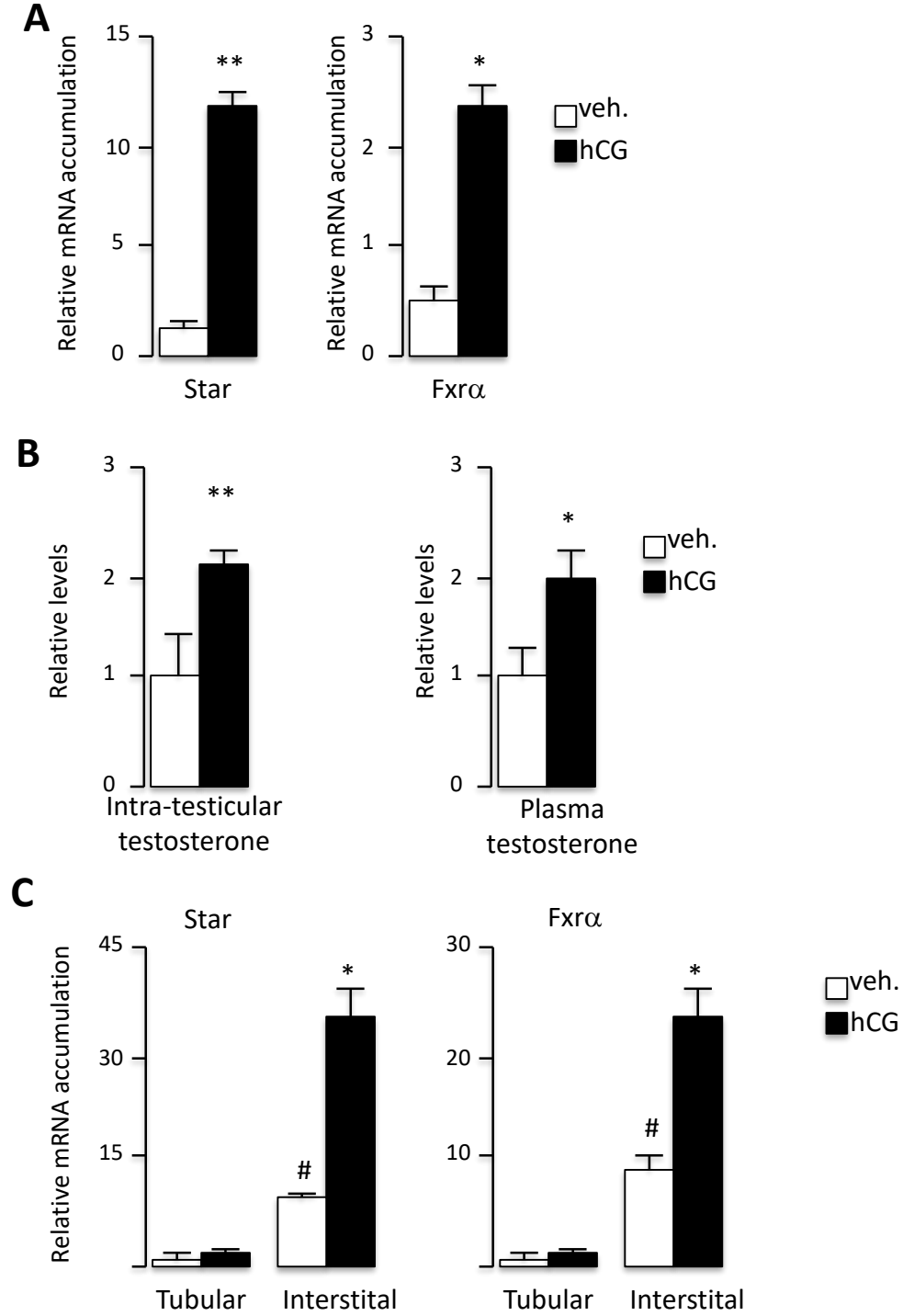
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### **Supplemental Figure 3.**

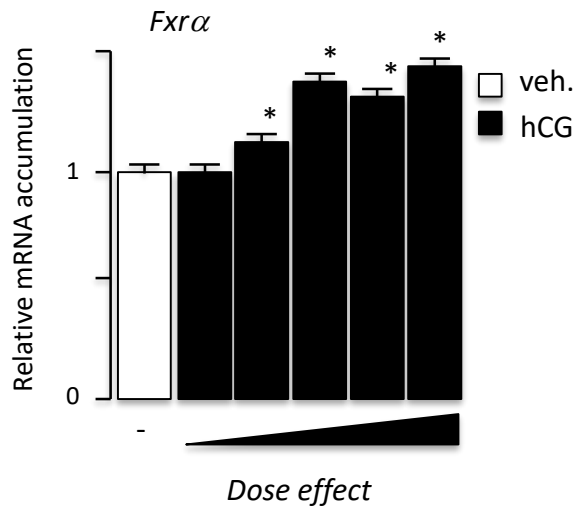
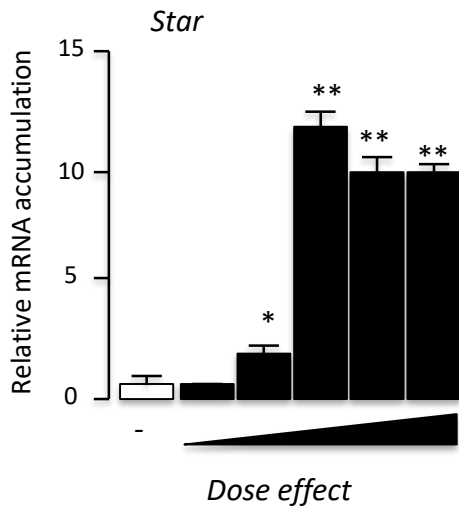
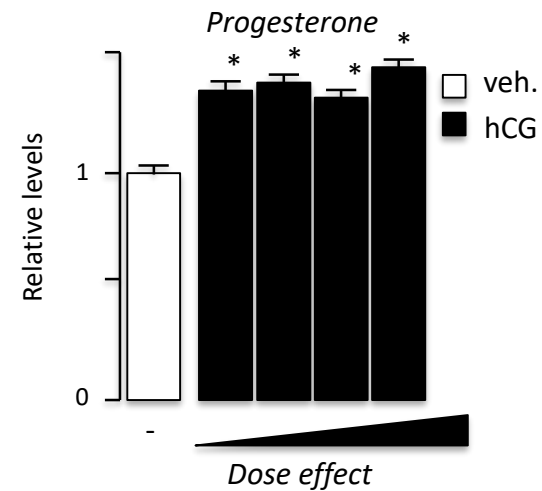
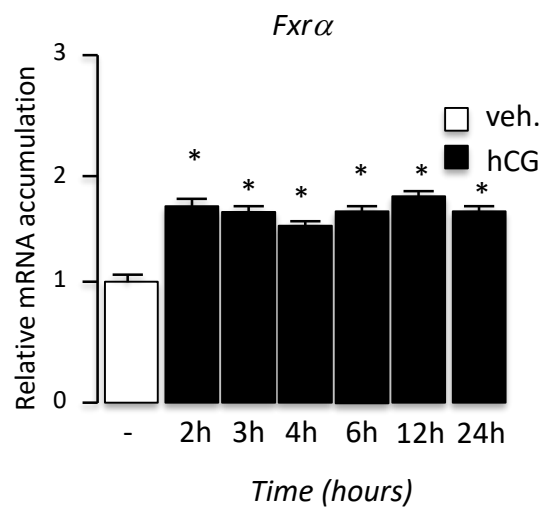
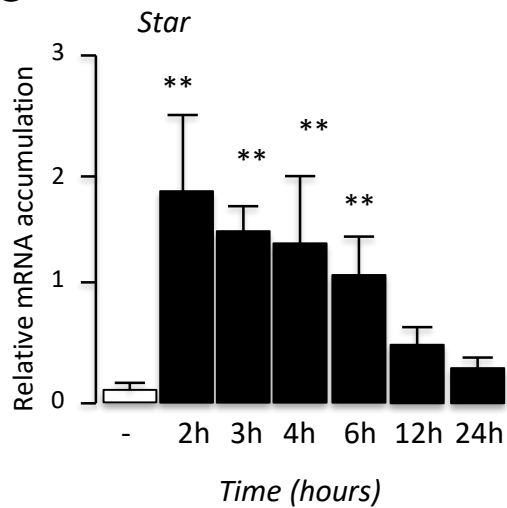
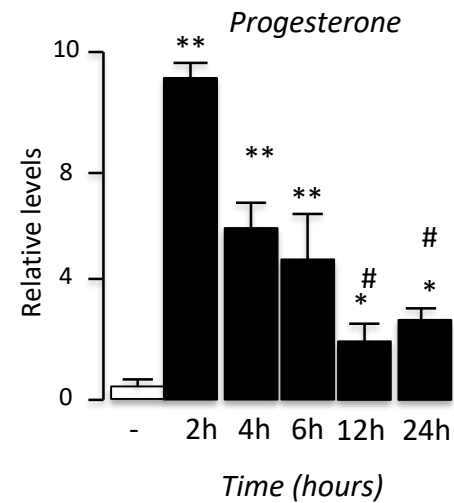
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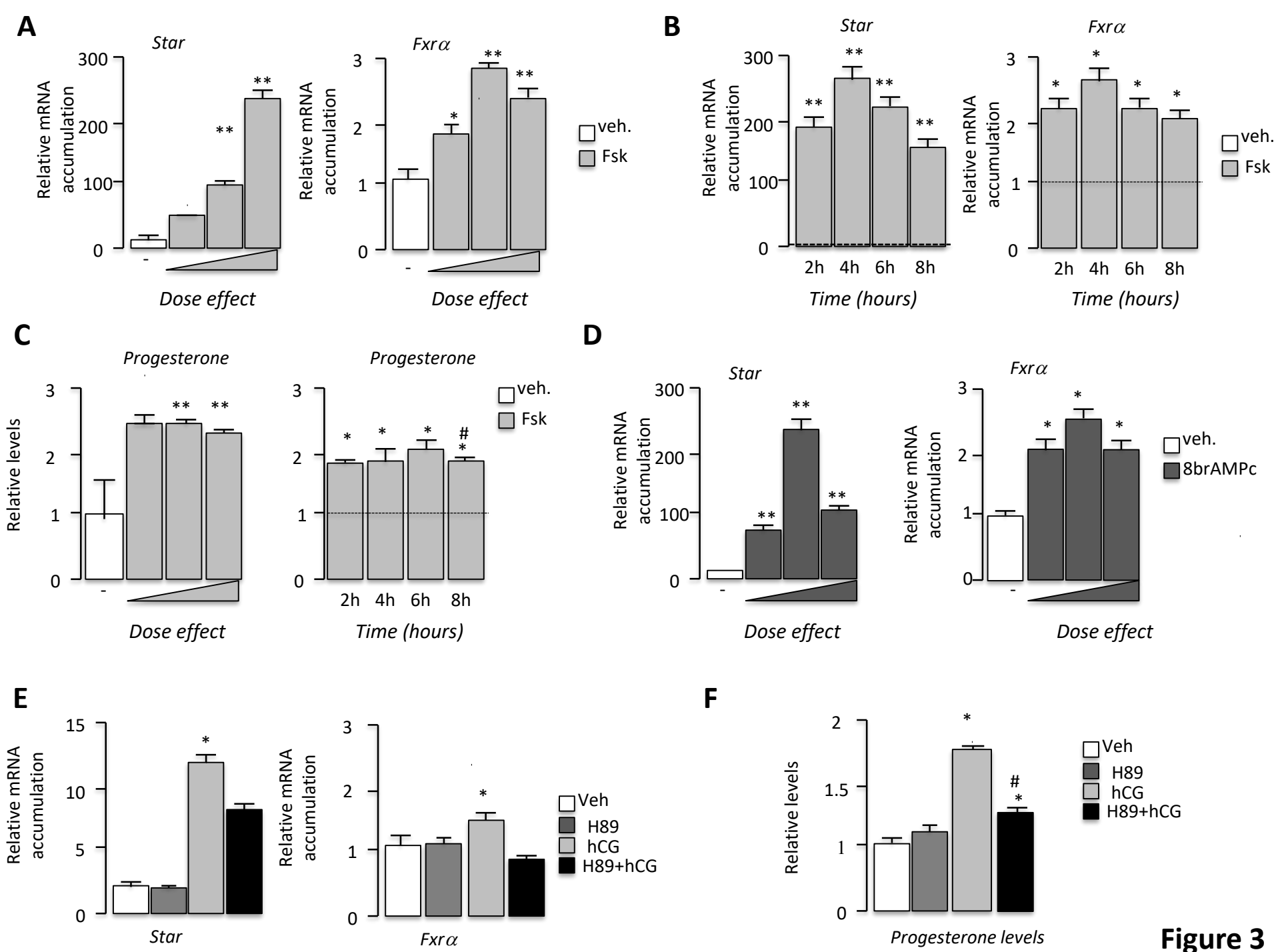
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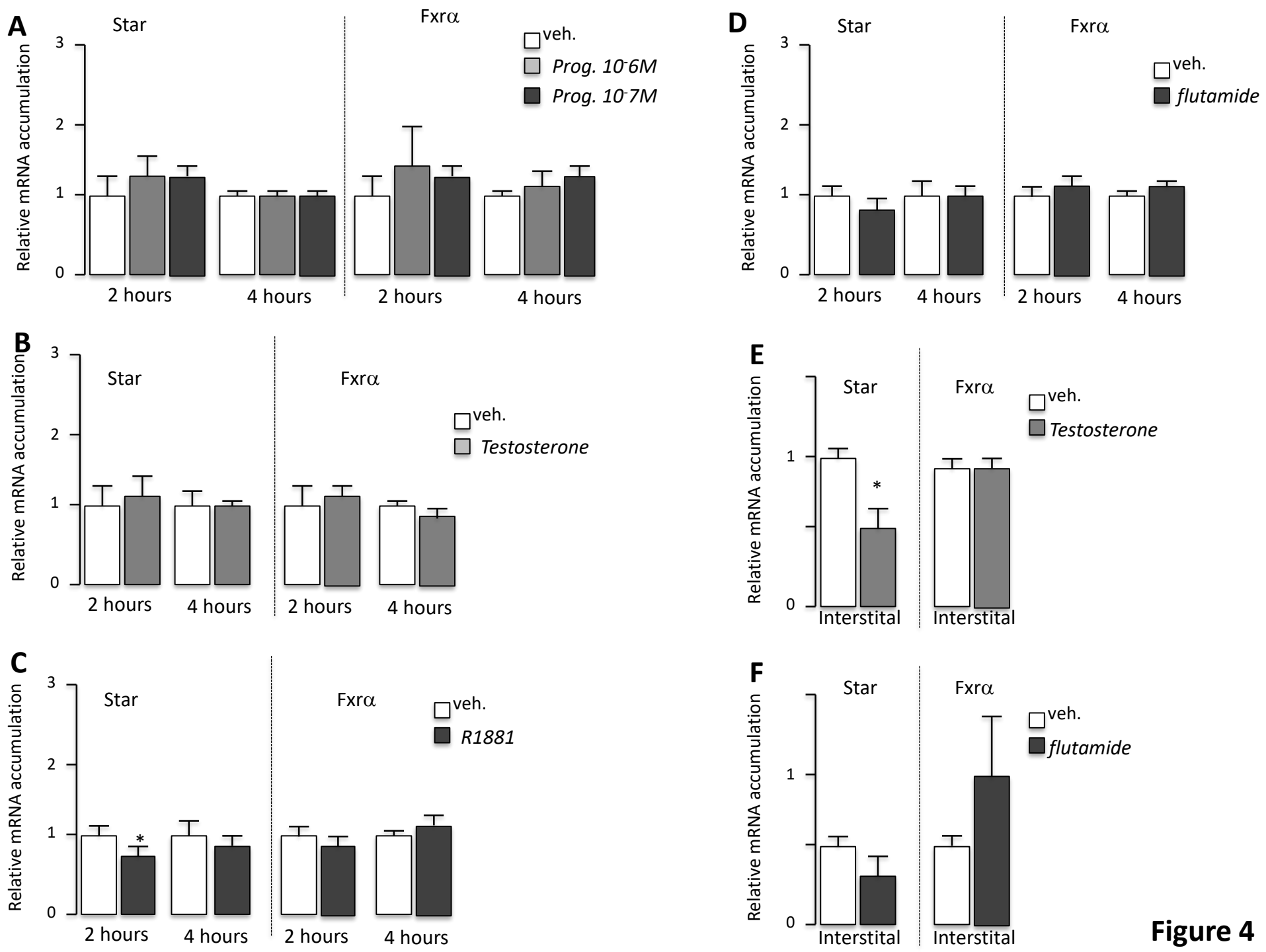




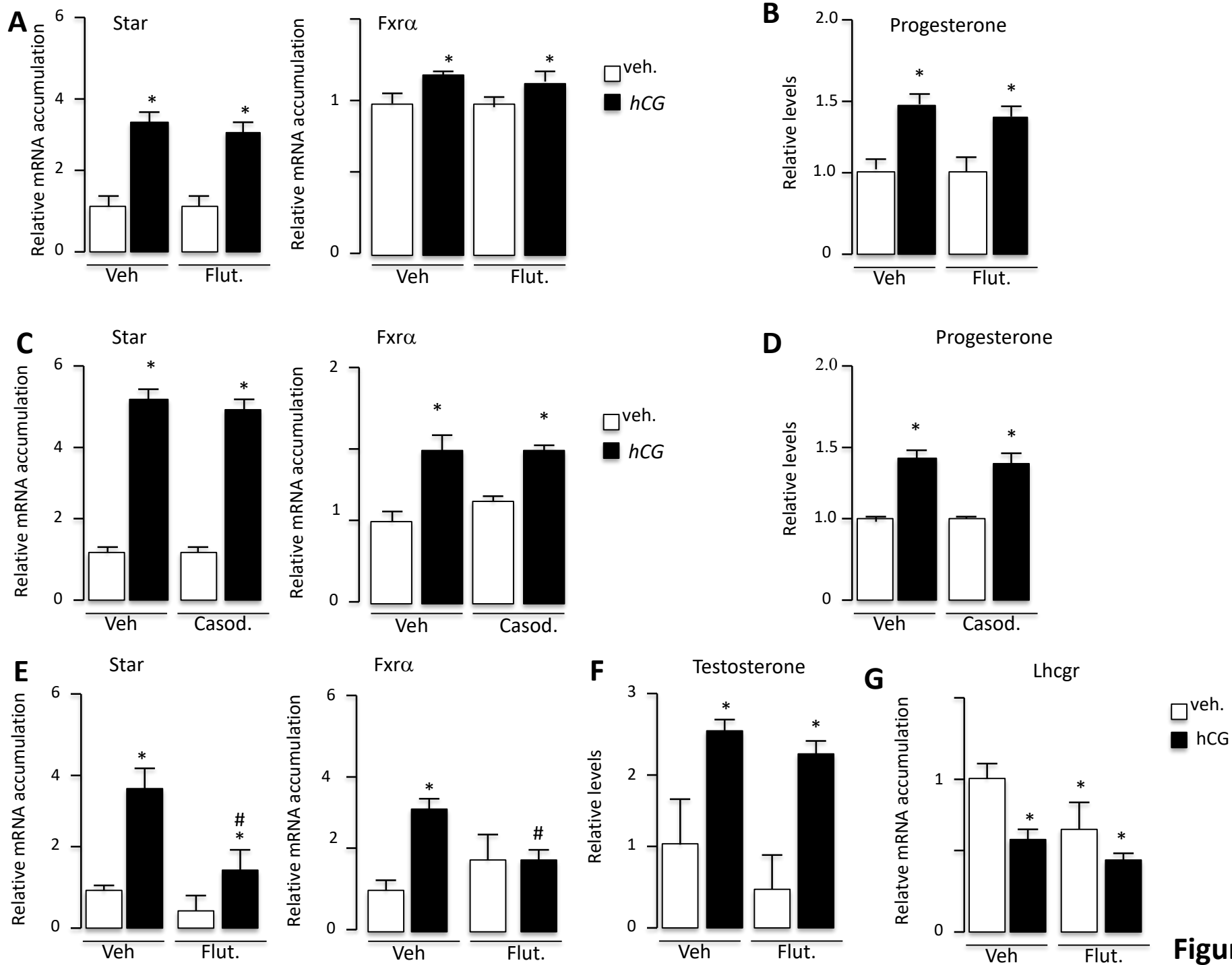
**Figure 1**

**A****B****C****D****Figure 2**

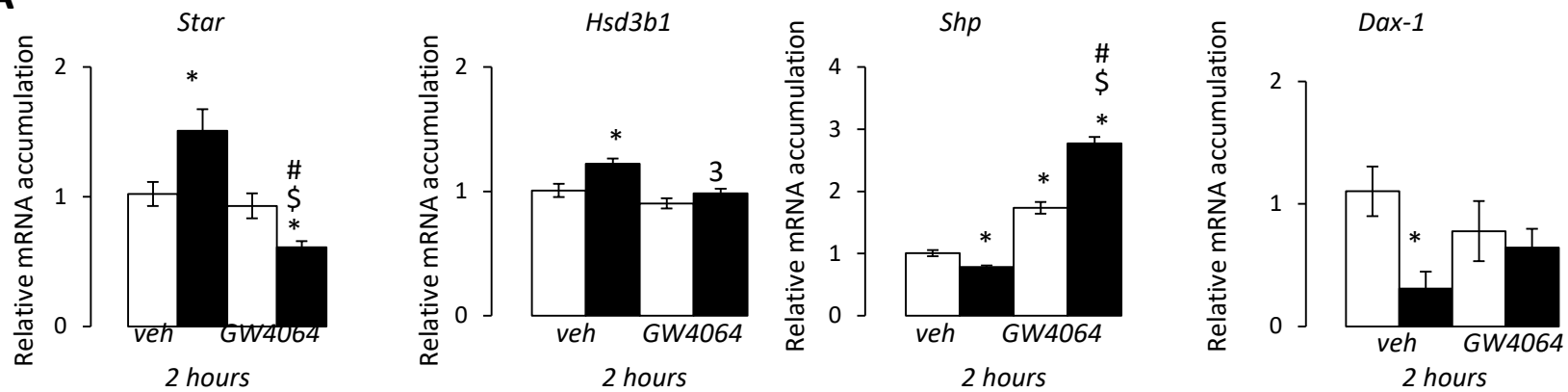
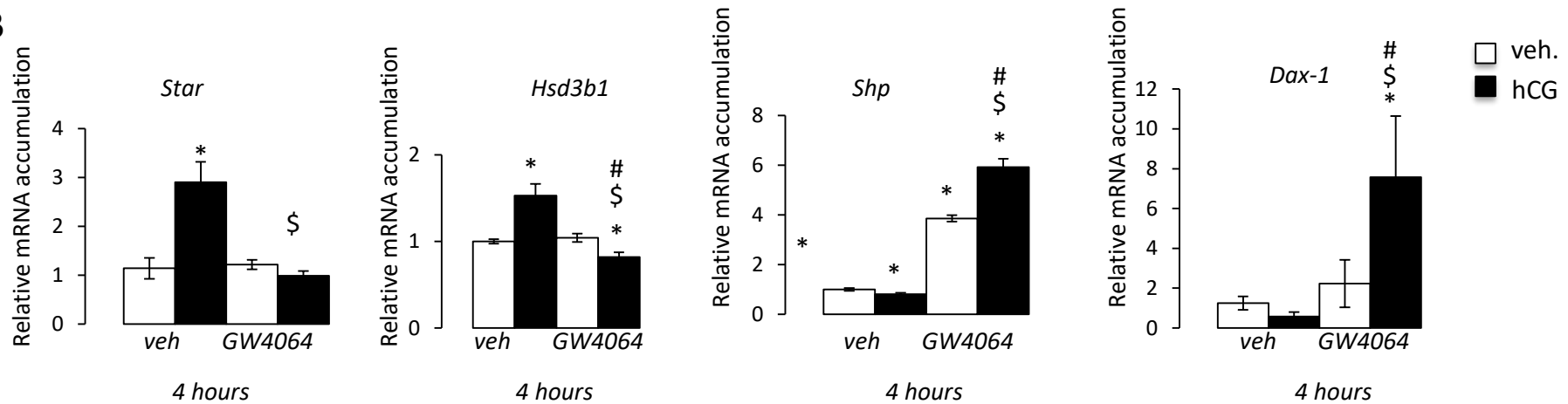
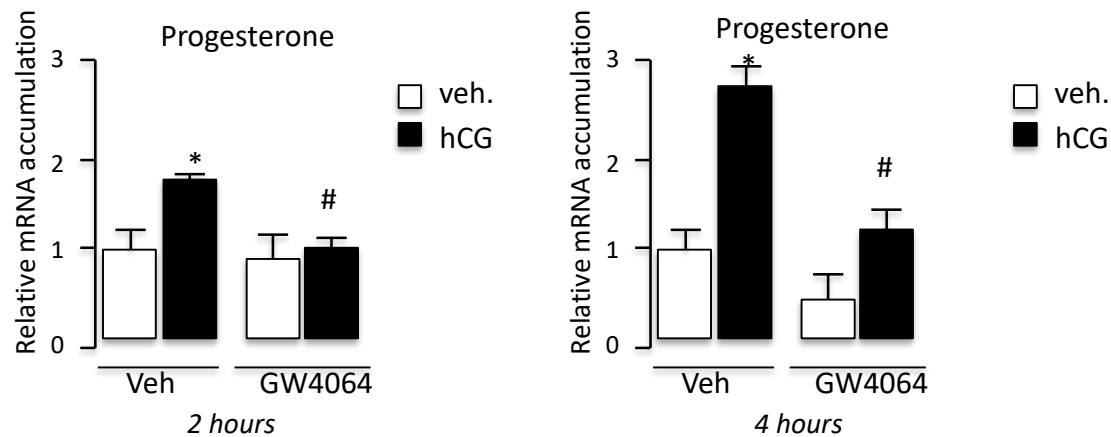


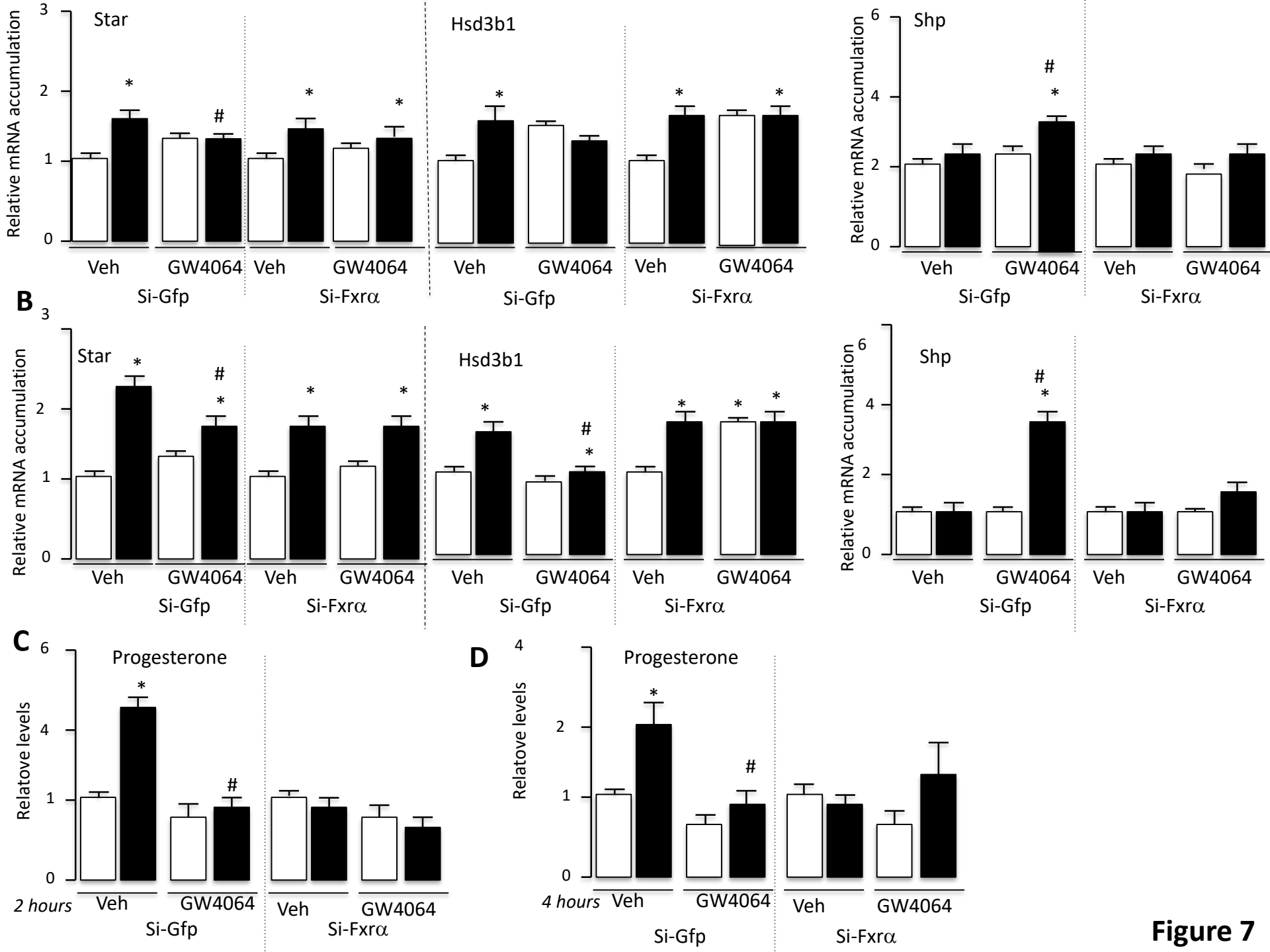


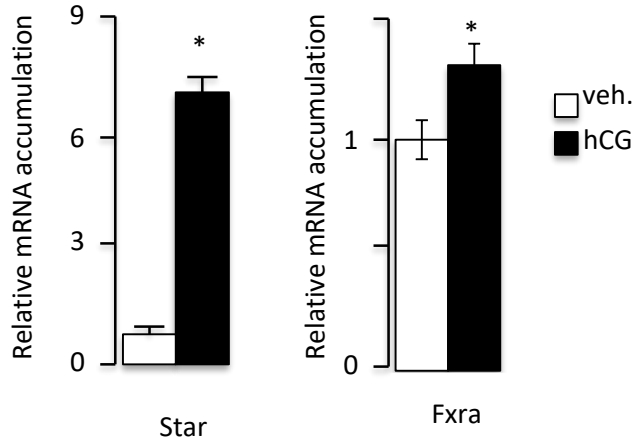
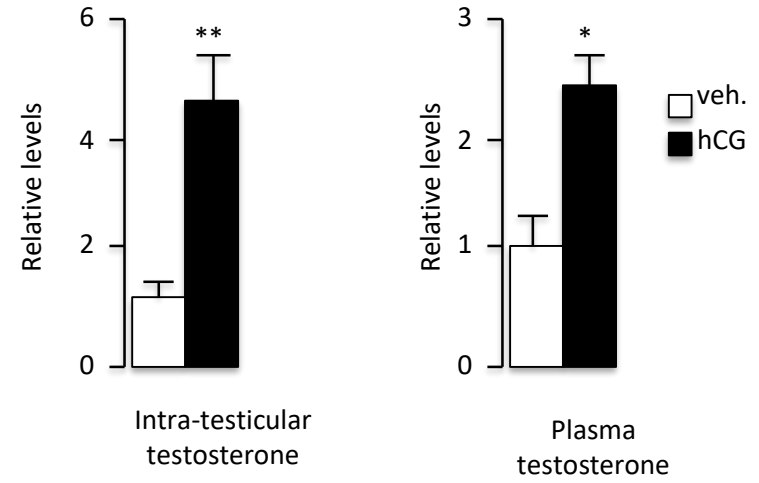
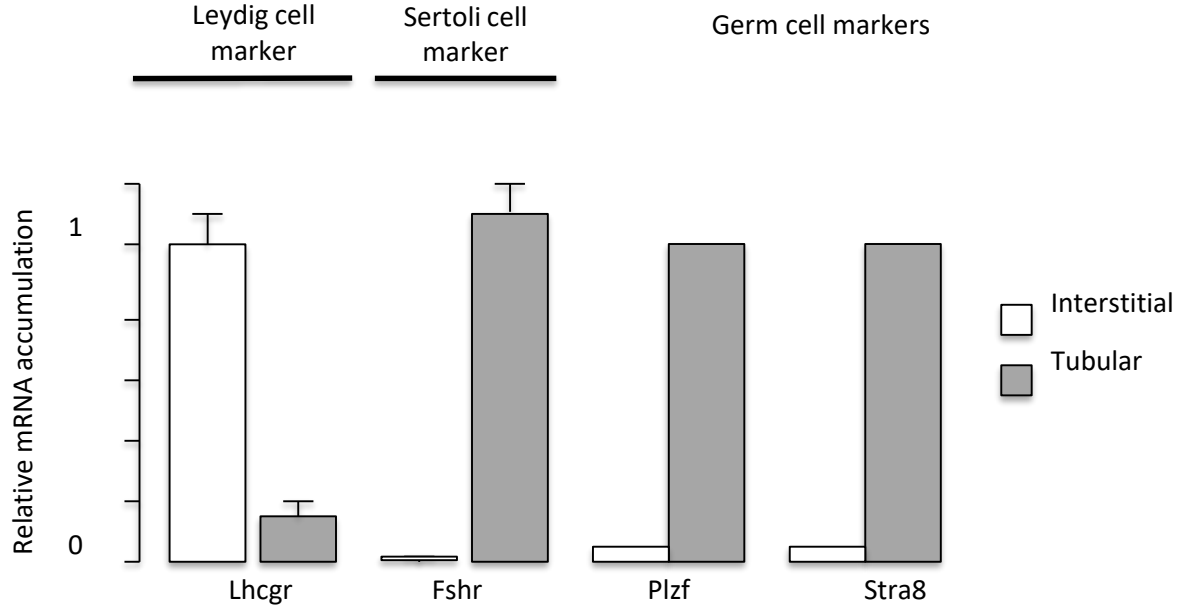
**Figure 4**



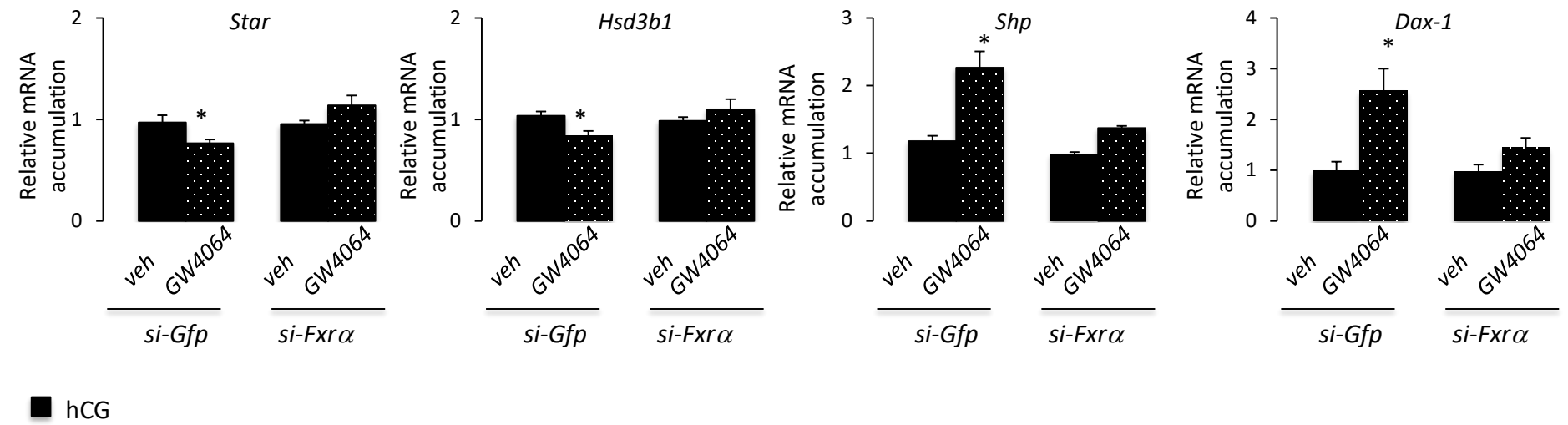
**Figure 5**

**A****B****C****Figure 6**



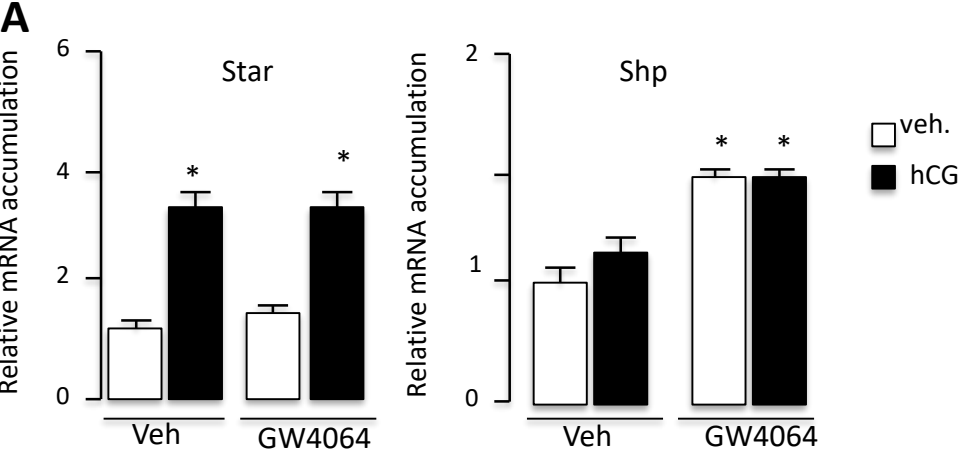
**A****B****A****Supplemental Figure 1**





**Supplemental Figure 2**

6h hCG + 4 hours GW4064



Supplemental Figure 3