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1)Title Page:

Testosterone augments FSH Signaling by upregulating the expression and activity of FSH-Receptor in Pubertal Primate Sertoli cells

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2) Abstract:

The synergistic actions of Testosterone (T) and FSH via testicular Sertoli cells (Sc) regulate male fertility. We have previously reported that the actions of these hormones (T and FSH) in infant monkey testes are restricted only to the expansion of Sc and spermatogonial cells. The robust differentiation of male Germ cells (Gc) occurs after pubertal maturation of testis. The present study was aimed to investigate the molecular basis of the synergy between T and FSH action in pubertal primate (*Macaca mulatta*) Sc. Using primary Sc culture, we here have demonstrated that T (but not FSH) downregulated *AMH* and *Inhibin-β-B*(*INHBB*) mRNAs in pubertal Sc. We also found that, prolonged stimulation of T in pubertal Sc significantly elevated the expression of genes involved in FSH signaling pathway like *FSH-Receptor (FSHR), GNAS* and *RIC8B,* and this was associated with a rise in cAMP production. T also augmented FSH induced expression of genes like *SCF, GDNF, ABP* and *Transferrin* (*TF*) in pubertal Sc. We therefore conclude that T acts in synergy with FSH signaling in pubertal Sc. Such a coordinated network of hormonal signaling in Sc may facilitate the timely onset of the first spermatogenic wave in pubertal primates and is responsible for quantitatively and qualitatively normal spermatogenesis.

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3) Introduction

Testosterone (T) and FSH are the major endocrine regulators of spermatogenesis which occur inside the specialized microenvironment of testis (Sharpe, 1994). Sertoli cells (Sc) are the principal somatic component of the testis which simultaneously express both FSH-Receptor (FSHR) and Androgen Receptor (AR) and thereby direct the regulated division, differentiation and survival of the male germ cells (Gc) upto sperm (Hai et al., 2014; Smith LB, 2015). AR is a ligand-inducible transcription factor that binds to the specific DNA sequences having *Androgen Response elements* (*ARE*) to initiate the androgen-dependent transcriptional events (Walker and Cheng, 2005). On the other hand, FSH-R is a G protein-coupled receptor (GPCR) which gets activated upon FSH binding and employs the membrane bound Adenylyl Cyclase (AC) to catalyze the production of cAMP via the intracellular G proteins, e.g., stimulatory $G\alpha_s$(GNAS) or inhibitory $G\alpha_i$ (GNAI) subunits (Walker and Cheng, 2005). FSH mediated cAMP pathway in Sc subsequently stimulate multiple signaling cascades such as PKA, PKC, PI3K, Akt/PKB and ERK1/ERK2 to induce the transcription of several genes like *Stem cell factor (SCF)*, *Glial derived neurotropic factor (GDNF)*, *Androgen binding protein (ABP)* and *Transferrin* (*TF*) etc (Walker and Cheng, 2005).

An intriguing phenomenon called “mini-puberty” occurs in neonatal and infant male primates (upto 3-4 months of age) where the circulatory levels of gonadotropins are comparable to that found during natural puberty (12-14 years and 3-4 years of age in boys and monkeys respectively) with prolific expansion of Sc and undifferentiated spermatogonia (Plant, 2015). Although such high level of gonadotropins fail to induce massive Gc differentiation and meiotic division in infant testes, this event plays critical role in masculinization of male brain and thereby regulates the pubertal timing for the robust spermatogenic initiation at later age during puberty

(Rey, 2014; Sharpe et al., 2003). It has been demonstrated earlier that in primate Sc, the ability of T to bind with AR and FSH induced cAMP production are compromised during infancy (Berensztein et al., 2006; Bhattacharya et al., 2015; Boukari et al., 2009; Lee et al., 1983; Majumdar et al., 2012; McKinnell et al., 2001; Rey et al., 2009). FSH, T, Thyroid hormone (T_3/T_4) and Retinoic Acid are considered to be the major regulator of functional maturation of pubertal rodent Sc (Buzzard et al., 2003; Holsberger et al., 2003; Sharpe et al., 2003; Walker, 2003; Wood and Walker, 2009).

Extensive studies from rodents to primates have confirmed the absolute requirement of androgen action via testicular Sc for the completion of meiotic division of Gc (Chang et al., 2013; Verhoeven et al., 2010). On the other hand, the critical role of FSH in male fertility is a controversial subject till date (Huhtaniemi, 2015; Nieschlag et al., 1999). Knock-out mouse models demonstrated that FSH is dispensable for spermatogenesis as such mice being fertile albeit presenting decreased testis size and partial spermatogenic failure (Abel et al., 2000; Dierich et al., 1998; Jonas et al., 2014; Kumar, 2007; Kumar et al., 1997). Men with an inactivating mutation in the FSHR, leading to decreased FSH binding and subsequent signaling, reveal variable degrees of spermatogenic abnormalities but not absolute male infertility (Tapanainen et al., 1997). In contrast, infertility is reported in men lacking normal circulating FSH (Phillip et al., 1998; Simoni and Casarini, 2014; Zheng et al., 2017). Furthermore, hCG driven depletion of circulatory FSH in adult men led to poor sperm counts, with one individual developing complete azoospermia, which was restored upon FSH supplementation (Matsumoto et al., 1986). A hypophysectomized man having complete gonadotropin deficiency and a mutated *FSHR* with constitutive FSH independent cAMP production by Sc was found to be fertile (Gromoll et al., 1996). Such a mutated *FSHR* has recently been shown to restore male fertility in

LH deficient mice without any support from androgen (Oduwole et al., 2018). FSH has also been reported to regulate the number of pachytene spermatocytes in adult humans (Matthiesson et al., 2006).

In various clinical and/or experimentally induced gonadotropin deficient primate models, either FSH (Bremner et al., 1981; Foresta et al., 1998; Gromoll et al., 1996; Marshall et al., 1995; Matsumoto et al., 1986, 1983; Simorangkir et al., 2009; Van Alphen et al., 1988; Weinbauer et al., 1991) or T (via LH or hCG)(Burris et al., 1988; Marshall et al., 1986, 1984, 1983; Matsumoto et al., 1984; Steinberger et al., 1973; Weinbauer et al., 1988) has been independently reported to reinitiate spermatogenesis. However, the restoration of the spermatogenic progression was significantly improved when both FSH and T (via LH) are supplemented together (Acosta et al., 1992; Arslan et al., 1993; Matthiesson et al., 2006; Schlatt et al., 1995). Furthermore, some clinical studies with human subjects suffering from *hypogonadotropichypogonadism* showed promising results in terms of testicular maturation and Gc differentiation when stimulated with FSH and hCG in combination (Burgués and Calderón, 1997; Kliesch et al., 1995; Kung et al., 1994). All these data together suggest that both FSH and T act in synergy to maximize the process of spermatogenic output (McLachlan et al., 2002; Ruwanpura et al., 2010). Furthermore, pulsatile treatment of LH and FSH in combination for 11 days have been shown to generate better impact on Gc differentiation with the appearance of differentiating spermatogonia B and primary spermatocytes as compared to independent treatment of either LH or FSH in juvenile (16 months of age) male monkeys (Ramaswamy et al., 2000). Finally, studies with double knock out mouse models for both *FSHR* and *AR* have now confirmed how this synchronization between FSH and T action plays a critical role in the spermatogenic progression and male fertility (Abel et al., 2008; O'Shaughnessy et al., 2012). However, the molecular crosstalk between T and FSH

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111 signaling during pubertal development of testis not yet been studied in primate Sc, mainly due to
112 requirement of suitable *invitro* Sc cultures from primate testis.

113 In the present study, we for the first time, have investigated the molecular basis of the synergistic
114 action of FSH and T or vice versa in cultured pubertal primate Sc which is critical for the robust
115 onset of spermatogonial differentiation (Sharpe et al., 2003). We have utilized primary culture
116 of Sc as successfully demonstrated by us before(Bhattacharya et al., 2015; Majumdar et al.,
117 2012) to compare the action of FSH and T in terms of gene expression either in isolation or in
118 combination in pubertal monkey Sc. Cultures of infant Sc were also employed for comparison
119 with pubertal Sc in this study, as infant Sc have already been shown to have a limited hormonal
120 (both T and FSH) responsiveness which is responsible for the spermatogenic quiescence of testis
121 at this phase of development (Bhattacharya et al., 2015; Majumdar et al., 2012).

122 **4) Materials and Methods**

123 *Animals*

124 Four juvenile (18-22 months old) and four infant (2-3 months old) male rhesus monkeys
125 (*Macaca mulatta*) were used. Monkeys were obtained from the Primate Research Centre of the
126 National Institute of Immunology (NII) where they were housed according to the guidelines of
127 the Committee for the Purpose of Control and Supervision of Experiments on Animals
128 (CPCSEA), Government of India, under a 12:12 hour (hr) light:dark cycle with food and water
129 provided ad libitum. The juvenile monkeys were surgically implanted with indwelling venous
130 catheters and maintained in remote infusion withdrawal cages, as previously described by us
131 (Bhattacharya et al., 2015; Devi et al., 2006; Majumdar et al., 2012). Bilateral castration was
132 performed as described previously (Bhattacharya et al., 2015; Devi et al., 2006; Majumdar et al.,

2012). Experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) of NII and CPCSEA.

Reagents

Recombinant monkey (rm) FSH (AFP6940), GnRH and an anti-cAMP antibody (Lot CV-27) were obtained from the National Hormone & Peptide Program (NHPP), NIH (Torrance, CA, USA). A polyclonal antibody to T (T3-125) was purchased from Endocrine Sciences Laboratories (Calabasas, CA, USA) and used for radioimmunoassay of this steroid. All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA) unless stated otherwise.

Induction of precocious puberty

Puberty was triggered precociously in the juvenile monkeys by eliciting premature endogenous gonadotropin secretion with an intermittent i.v. infusion of GnRH (0.15 µg/min for 2 min every 3 hour) administered for 4-5 weeks as previously described (Bhattacharya et al., 2015; Devi et al., 2006; Majumdar et al., 2012). Plasma T levels were tracked in weekly samples and animals were castrated after peak levels of this steroid reached a range comparable to that observed in pubertal rhesus monkeys. Sc from these testes were subsequently referred to as “pubertal” Sc. This model of puberty was employed because it enabled the testes from different monkeys to be harvested at a synchronized stage of pubertal development and also at a time before the appearance of a large number of advanced Gc, which are known to interfere with the isolation of Sc (Bhattacharya et al., 2015; Devi et al., 2006; Majumdar et al., 2012).

Isolation and culture of Sc

Sc from infant and pubertal monkeys were isolated by sequential enzyme digestion with collagenase and pancreatin, as described by us previously (Bhattacharya et al., 2015; Devi et al., 2006; Majumdar et al., 2012, 1998). The cells were then suspended in Dulbecco Modified Eagle Medium/Nutrient Mixture F-12 HAM (DMEM/F12 HAM) containing 1% fetal calf serum (FCS). Equal numbers of cell clusters (0.5×10^4 clusters/well) were seeded in each well of a 24 well culture plate (day 1) and cultures were kept at 34°C in a 5% CO_2 incubator. On Day 2, cells were washed and maintained in media containing 1% serum replacement growth factors (GF), containing Sodium selenite, 5 µg/ml; Insulin, 10 µg/ml; Transferrin, 5 µg/ml; and Epidermal growth factor 2.5 ng/ml. This GF media was replaced every 24 hour (hr). On day 4 of culture, Sc were treated with 20mM of Tris-HCl at pH= 7.4, to remove residual Gc and Sc culture were maintained by 1% GF for next 24hr before initiating the *in vitro* hormonal stimulations. We have previously reported that cultured monkey Sc were highly pure and maintain hormone responsiveness for 5 to 6 days with GF media (Bhattacharya et al., 2015; Devi et al., 2006; Majumdar et al., 2012, 1998).

Evaluation of Sc culture purity

On Day 5 of culture, cell viability was determined by trypan blue exclusion. Leydig cells (Lc) contamination was evaluated by cytochemical staining for 3β-hydroxysteroid dehydrogenase (3β-HSD) activity (Klinefelter et al., 1987). A crude preparation of monkey Lc, which was obtained while isolating Sc from testis of pubertal monkeys, was used as a positive control to detect 3β-HSD activity. Peritubular myoid cells (PTc) and Sc were identified by staining for alkaline phosphatase activity and Oil Red-O, respectively (Chapin et al., 1987).

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In vitro Treatments

On day 5 of culture, cells were exposed for next 24 hr to i) GF media alone (as vehicle or control) ii) vehicle containing either T (100 nM) or synthetic androgen R1881 (100 nM) [pubertal Sc were stimulated by both of the androgens, whereas infant Sc were treated only with T, due to limited cells],iii) vehicle containing rmFSH (5ng/ml), and iv) vehicle containing rmFSH (5ng/ml) and T or R1881 (100 nM) in combination all in the presence of Isobutyl-Methyl-Xanthine (IBMX, 0.1 mM) for hormone mediated gene expression studies. At the end of culture, Sc were washed with calcium free buffer and then dissociated from surface of plate using trypsin-EDTA. After washing, cells were lyzed in Tri reagent and stored at -80˚C before extraction of RNA. Extracted RNA was treated with DNase I to remove trace DNA contamination, and DNase I was heat inactivated (Ambion Inc., Austin, TX, USA) and converted to cDNA as described by us previously (Bhattacharya et al., 2015; Majumdar et al., 2012).

In order to estimate cAMP production by Sc, cultured cells were treated for 1/2hr and 24hr with i) GF media only (as vehicle or control), ii) GF media either containing T (100 nM) or synthetic androgen R1881 (100 nM) [pubertal Sc were stimulated by both of the androgens, whereas infant Sc were treated only with T, due to limited cells], iii) vehicle containing rmFSH (5ng/ml) and iv) vehicle containing rmFSH (5ng/ml) and T (100 nM) to) in combination, all in the presence of Isobutyl-Methyl-Xanthine (IBMX, 0.1 mM). At the end of culture, media were collected and boiled prior to storage at -80°C.

Similarly, to evaluate the E_2 production, cultured Sc were treated for 24hr with T (100 nM) and rmFSH (5ng/ml), either alone or in combination, or with media alone (as vehicle or control), all

195 in the presence of IBMX(0.1 mM). At the end of culture, media were collected for storage at -
196 80°C.

197 The doses of rmFSH, 8-Br-cAMPand androgens (either T or R1881) used here was found to be
198 bioactive in cultures of monkey Sc as described by us previously (Majumdar et al., 2012). For
199 clarity, the experimental flow chart is presented in **Table 1**.

200 *Cyclic AMP ,E_2 and T assay*

201 Radio-immunoassay (RIA) was employed to measure T levels in plasma of monkeys and cAMP
202 and E_2 concentrations in culture medium as described by us previously (Bhattacharya et al.,
203 2015; Majumdar et al., 2012). The intra and inter assay coefficient of variations were less than 5
204 and 9% respectively for E_2 evaluated from media exposed to Sc culture for 24hr. Similarly, the
205 intra and inter assay coefficient of variations were less than 7 and 10% respectively for T
206 evaluated from plasma of infant, juvenile and pubertal (GnRH stimulated juveniles) monkeys.

207 *Quantitative Real-time-PCR (qRT-PCR) analyses*

208 Quantitative-RT-PCR amplifications were performed in a 96-well plate in a Realplex$_S$ PCR
209 machine (Eppendorf, Germany) in a total volume of 10 µl [1 or 2 µl of cDNA (depending upon
210 the abundance of the transcripts), 0.5 µM of each primer and 5 µl of Power SYBR Green Master
211 Mix (Applied Biosystems). Primers for each gene (Supplementary Table S1) were independently
212 validated by a standard curve calculated from the serial dilutions of the cDNAs. The q-RT-PCR
213 reaction was initiated with melting of cDNA at 95°C for 15 min, followed by 40 amplification
214 cycles (15 sec at 95°C, 45 sec at 60°C and 45 sec at 65°C). Melting curve analyses for each gene
215 were performed immediately after the amplification to detect the specific amplification peak for
216 each gene. Expression of mRNA of the target genes were determined by the efficiency-corrected

ΔCt method [quantity = $(\text{Efficiency}+1)^{-Ct}$] as described by us earlier (Bhattacharya et al., 2015; Majumdar et al., 2012). For each sample, the relative quantity of each target gene was calculated by normalization with the relative quantity of the endogenous control *RPL32*. For each gene, the Mean±SEM of at least 3 individual experiments (i.e. 3 animals in each of the two age groups) were determined for each treatment group. The Primers are listed in **Supplemental Table S1.**

Presentation of data and statistical analysis

Mean values of the relative mRNA levels were derived from at least 3 independent Sc cultures (from at least 3 different monkeys, n=3) for each age group. For determination of cAMP or E_2 production and gene expression each treatment for a given monkey was examined from at least three independent wells. The average values (from replicate wells) were calculated and used to determine the overall mean for that particular infant or pubertal monkey. Mean of triplicates for a given treatment (for each monkey) was used to compare differences from other treatments in that particular monkey. Such means from at least 3 different infant or 3 different pubertal monkeys were used for determining the standard error within a particular developmental age group. All values were plotted as Mean with error bar for standard error of mean (Mean±SEM). Statistical significance was calculated by multiple measures ANOVA followed by Dunnett's post-test for comparing between treated with control groups, and Student t-test was performed to compare between any two treated groups. P value for significant difference in histograms is mentioned in legends to the graphs. Graphpad prism version 5.0 statistical program (254 Graphpad Software, Inc. San Diego, CA, USA) was employed for gene expression studies and for evaluation of cAMP/E_2 production as reported by us earlier to determine the significance of differences between means (Bhattacharya et al., 2015; Devi et al., 2006; Majumdar et al., 2012).

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5) Results

Preparation of pubertal monkeys and culture of pubertal and infant Sc.

Puberty was uniformly induced by pulasatile GnRH infusion in all the juvenile monkeys as described by us previously (Bhattacharya et al., 2015; Majumdar et al., 2012). Plasma levels of T in GnRH treated juvenile monkeys increased remarkably after 4-5 weeks of pulsatile GnRH stimulation from 0.22-0.87 ng/ml to reaching peak concentrationof6.9- 9.2 ng/ml,similar to that found during natural puberty. **Supplemental Table S2**shows the plasma T concentration of infant and pubertal monkeys used in this study. At the end of GnRH treatment, testicular size increased approximately 3-4 fold. Such hormone induced testes displayed enlargement of seminiferous tubules and initiation of Gc differentiation, as reflected by the presence of abundant type B spermatogonia and occasionally, primary spermatocytes, similar to puberty as to shown by us before (Bhattacharya et al., 2015; Majumdar et al., 2012). Sc from such monkeys were used as source of pubertal Sc. Infant and pubertal Sc formed confluent monolayers in culture with about 95% purity as indicated by Oil Red O staining (**Supplemental Fig S1**). Viability of cells on day 5 of culture was 98%. Although PTc specific alkaline phosphatase activity was present occasionally, contamination of PTc was < 2%, Lc were absent, as indicated by the lack of 3β-HSD activity in the cultured cells as shown in our previous studies (Devi et al., 2006; Majumdar et al., 2012).

Androgen mediated gene expression in Sc

On day 5 of culture, both the pubertal and infant Sc were stimulated with vehicle alone (as control) or vehicle containing either T/R1881or FSH (alone or in combination) for 24hr and the expression of *Inhibin-β-B* (*INHBB*), *AMH*, *Cytokeratin18* (*CK18*) and *Claudin11* (*CLDN11*)

mRNAs were evaluated by qRT-PCR analysis. Our results suggested that T or R1881 significantly ($p<0.05$) downregulated the expression of *INHBB* and *AMH* (**Fig. 1 A1&B1**) in pubertal Sc, however such T mediated action was not observed in infant cells (**Fig. 1 A2&B2**). Any such significant ($p<0.05$) downregulation in *CK18* expression was not observed in either pubertal or infant Sc with androgen stimulation (**Fig. 1 C1&C2**). Consistent with our previous observation (Majumdar et al., 2012), androgen (T or R1881) significantly ($p<0.05$) upregulated the mRNA expression of *CLDN11*, a component of blood testes barrier (BTB) in pubertal Sc (**Fig. 1 D1 and D2**). However, FSH either alone or in combination with T upregulated the expression of *INHBB* only in pubertal Sc, but not during infancy (**Supplemental Fig S2. A1 and A2**). This observation also supported by previous *in vivo* and *in vitro* reports (Rey, 2014). Consistent with previous reports (Edelsztein et al., 2016) we also found that the expression of *AMH* was upregulated by FSH alone in both infant and pubertal Sc, although such elevated expression level was not observed in pubertal cells when they were stimulated with FSH and T in combination (**Supplemental Fig S2. B1 and B2**). No significant ($p<0.05$) change in the expression level of *CK18* was observed with FSH stimulation without or with T in both pubertal and infant Sc (**Supplemental Fig S2. C1 and C2**).FSH significantly ($p<0.05$) upregulated the mRNA expression of *CLDN11*in pubertal Sc without or with the presence of T, unlike in infant Sc (**Supplemental Fig S2. D1 and D2**).

Effect of FSH and T on AR expression in Sc

FSH or T either alone or in combination, failed to show any significant change in the expression pattern of *AR* mRNA in both infant and pubertal Sc (**Supplemental Fig S3**).

T mediated augmentation of FSH induced gene expression associated with FSH signaling in pubertal Sc

On day 5 of culture, Sc of both the age groups were stimulated with vehicle alone or vehicle containing either FSH alone or FSH in combination with T for 24hr and the expression of genes involved in FSH signaling e,g. *FSHR*, *GNAS*, *RIC8B* mRNAs were evaluated by q-RT-PCR analysis. FSH stimulation resulted into an augmentation in expression of *FSHR* (**Fig. 2. A**), *GNAS* (**Fig. 2. B**), and *RIC8B* (**Fig. 2. C**) mRNAs in pubertal Sc, which were not observed in infant cells (**Fig. 2. D, E & F**). However, T supplementation along with FSH further showed a significant ($p<0.05$) additive effect in terms of the expressions of these genes as compared to either control or FSH alone in pubertal Sc only (**Fig. 2.A-C**). However, no such effects were observed in infant Sc (**Fig. 2. D-F**).

Androgen directly upregulates the expression of genes associated with FSH signaling in pubertal Sc

Cultured Sc of both the age groups were stimulated with vehicle alone or vehicle containing T or R1881 for 24hr and the expression of genes involved in FSH signaling e,g. *FSHR*, *GNAS*, *RIC8B*, *GNAI1*, *GNAI2*, *GNAI3*, *RGS-PX1* and *EPB41L2* mRNAs were evaluated by q-RT-PCR analysis. Unlike infant Sc, pubertal Sc showed a significant ($p<0.05$) rise in the transcripts of *FSHR* (**Fig. 3. A1&A2**), *GNAS* (**Fig.3. B1&B2**),*RIC8B* (**Fig. 3. C1&C2**) mRNAs with T or R1881 stimulation for 24hr. However, no such significant ($p<0.05$) changes in the transcription level were detected for *GNAI1* (**Supplemental Fig S4. A**), *GNAI2* (**Supplemental Fig S4. B**),*GNAI3* (**Supplemental Fig S4. C**), *RGS-PX1* (**Supplemental Fig S4. D**) and *EPB41L2* (**Supplemental Fig S4. E**) mRNAs either in pubertal or in infant cells with T treatment.

E_2 production by Sc at 24hr

In order to evaluate the aromatizable ability of Sc, cells obtained from both the age groups were stimulated with vehicle alone (as control) or vehicle containing either T or FSH (alone or in combination) for 24hr and the Sc exposed media were employed for E_2 assay. Although T were significantly ($p<0.05$) converted to E_2 by both pubertal and infant Sc, there were no remarkable impact of FSH in this process (**Supplemental Fig S5. A & B)**. Furthermore, there was no significant ($p<0.05$) developmental difference in the aromatizable ability of Sc found during infancy to puberty (**Supplemental Fig S5. A & B**).

Androgen mediated augmentation in cAMP production at 24hr in pubertal Sc

Sc obtained from both the age groups were stimulated with vehicle alone (as control) or vehicle containing either T/R1881 or FSH (alone or in combination) for 1/2 hr and 24hr and the Sc exposed media were employed for cAMP assay. Both at 1/2 hr or 24hr of time point, infant and pubertal Sc showed a significant rise ($p<0.05$) in cAMP production with FSH stimulation (without or with T) as compared to that of the controls (vehicle only) (**Fig. 4. A- D**). However, the magnitude of cAMP production was more prominent in the pubertal cells at 24hr (**Fig. 4. A**). No such significant rise in cAMP was observed by androgen (T or R1881) stimulation in both the age groups at 1/2 and 24 hr (**Fig. 4. A-D**). Furthermore, only in pubertal Sc, FSH and T showed an additive effect on cAMP production at 24hr (**Fig. 4. A**). Such additive effect of hormones (FSH and T) for cAMP production at 24hr was not even observed at 1/2hr in pubertal Sc **(Fig. 4. B)**. No significant ($p<0.05$) rise in cAMP production was discernible in infant cells in a time dependent (1/2hr to 24hr) manner (**Fig. 4. C& D**). P values from statistical analyses between different groups shown in **Fig. 4. A-D** provided in **Supplemental Table S3.**

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Androgen mediated augmentation in FSH responsive gene expression in pubertal Sc

In order to evaluate any synergistic action of T on FSH induced gene expression or vice versa, Sc obtained from both the age groups were treated with vehicle alone (as control) or vehicle containing either T or FSH (alone or in combination) for 24hr and the expression of the genes responsive towards FSH (*SCF, GDNF, ABP* and *Transferrin*) were investigated. We have found that in pubertal cells, FSH alone significantly ($p<0.05$) augmented the expression of *SCF, GDNF, ABP, Transferrin* mRNAs, whereas T independently upregulated the transcriptions of *ABP, Transferrin* (**Fig. 5. A-D**). It is essential to note here that, the degrees of rise in the transcripts of *SCF, GDNF, ABP* and *Transferrin* were significantly ($p<0.05$) augmented further when FSH was supplemented with T as compared to either control, FSH or T alone in the pubertal cells (**Fig. 5. A-D**). On the other hand, infant Sc did not show any kind of hormonal responsiveness either by FSH or T alone or in combination for all the genes studied (**Fig. 5. E-H**). The expression of *CLDN11* mRNA was found to be uniform by T or R1881 stimulation in combination with FSH in pubertal Sc (**Supplemental Fig S6**).

6) Discussion

The present study was aimed to investigate the molecular events responsible for the synchronized action of T and FSH signaling during pubertal maturation of Sc which is critical for establishing the male fertility. Data obtained from pubertal Sc were compared with infant monkey Sc which are poor responders to androgen and FSH (Bhattacharya et al., 2015; Majumdar et al., 2012). We induced puberty in juvenile rhesus monkeys by intermittent infusion of GnRH into circulation, which is known to activate the endogenous gonadotrophin secretion and spermatogenesis prematurely (Bhattacharya et al., 2015; Majumdar et al., 2012). Such a

model of induced puberty allows harvesting Sc from different monkeys, precisely at similar time during the onset of spermatogenesis, reducing individual variations. It further helps in isolation of Sc from testis at a stage prior to massive accumulation of Gc (like adults)which interferes with the isolation process (Devi et al., 2006; Majumdar et al., 1998).

The lack of T responsiveness by infant Sc in terms of *AMH, INHBB, CK18* and *CLDN11*expressionsgetsupport from previous reports showing poor translation and function of AR during this stage of development (Berensztein et al., 2006; Boukari et al., 2009; Chemes et al., 2008; McKinnell et al., 2001; Rey et al., 2009). *AMH, INHBB, CK18* and *CLDN11* are well known maturation markers of Sc (Hai et al., 2014; Sharpe et al., 2003). Expression of AMH is known to be high in infant primate Sc including human male babies and progressively decline with pubertal maturation of the testes (Edelsztein et al., 2016). Our data revealed that androgen (both T and R1881) stimulation led to the downregulation of *AMH* and *INHBB* xpression in pubertal Sc. T and LH are reported to downreguate the expression of *AMH* (Edelsztein et al., 2016) and *INHBB*(Ramaswamy et al., 2003)in maturing pubertal testes respectively. Furthermore, such T mediated pubertal upregulation of *CLDN11* mRNA observed in this study was also consistent with our previous observation (Majumdar et al., 2012). Moreover, clinical data show that persistent expressions of either *AMH* or *CK18* in adult Sc are associated with spermatogenic failure (Steger et al., 1999; Young et al., 1999). Therefore, these data together established that the pubertal Sc used in this study were sufficiently “mature” to show potential effect of androgen (T or R1881) *in vitro*. Additionally, this observation also provided necessary explanation for the appropriateness of the use of cultured Sc in this study. It is essential to note here that, T the natural androgen of testis, was preferred in this study over dihydro-testosterone (DHT), an analogue of T synthesized in the male reproductive tract. Furthermore, unlike pubertal

cells , since the yield of isolated Sc from infant testes was low only natural testicular androgen (T) treatment was favored over synthetic R1881 in this group.

FSH has been shown to act as a negative regulator of T mediated ERK kinase activity in rat Sc (Shupe et al., 2011). In pubertal rat testes, the expression of *AR* mRNA has been reported to be regulated by androgens in a stage specific manner *in vivo*(Bremner et al., 1994; Shan et al., 1997; Zhu et al., 2000). Furthermore, although T has no impact on *AR* mRNA expression in cultured rat Sc (Blok et al., 1992), combined FSH and T treatment have been shown to give an additive effect on *AR* mRNA expression in rat Sc *in vitro*(Verhoeven and Cailleau, 1988). However, in our study, *AR* mRNA expression was also found to be unaltered with T or FSH stimulation either alone or in combination in both infant and pubertal cells.

FSH has been reported to modulate *FSHR* mRNA expression in cultured rat Sc in a time dependent manner (Walker and Cheng, 2005). Long term FSH exposure (for 24hr) has been shown to upregulate *FSHR* expression in rat Sc *in vitro* (Viswanathan et al., 2009). We have previously reported that FSH selectively augments the expression of *FSHR*, *GNAS* ($G\alpha_{s,}$) , stimulatory G protein subunit associated with FSHR, and *RIC8B*, a Guanine nucleotide Exchange Factor (GEF) that activates $G\alpha_s$ subunit by replacing bound GDP with free GTP in pubertal Sc (Bhattacharya et al., 2015). In the present study, our data demonstrated that the expressions of such FSH induced genes were augmented further when FSH was supplemented with T treatment to pubertal Sc. However as expected, infant Sc remained unresponsive towards such hormonal stimulation. This data indicated that unlike in infant Sc, T and FSH signaling show a positive synergistic effect on gene expression in pubertal Sc. To delineate the molecular basis of such effect of hormonal action in pubertal Sc, androgen mediated expression of *FSHR*, *GNAS*, and *RIC8B* mRNAs were investigated further. Our data suggested that prolonged

exposure (for 24hr, not at 1/2hr) of androgen (T or non-matabolizableR1881) to pubertal Sc resulted a significant augmentation in FSH signaling. Unlike infant Sc, androgen (T or R1881) directly upregulated *FSHR*, *GNAS*, and *RIC8B mRNA* expressions in pubertal Sc. Furthermore, T also potentially augmented FSH induced cAMP production (as compared to that of the FSH treatment alone) in pubertal cells at 24hr, which was not observed in infant Sc.

Androgen upregulates *FSHR* mRNA in murine granulosa cells to play crucial role in follicular development (Sen et al., 2014). Androgen is reported to act via both classical and non- classical pathway in rat Sc (Toocheck et al., 2016). In non-classical pathway, T binding to AR rapidly activates Src tyrosine kinase in a cAMP independent manner and subsequently activates the ERK-MAPK pathway (Cheng et al., 2007) to upregulate the transcription of *cAMP response element-binding protein (CREB)*, which is maintained even at 24hr in pre-pubertal rat Sc (Fix et al., 2004). Additionally, androgen is reported to induce intracellular release of Ca^{+2} in rat Sc (Gorczynska and Handelsman, 1995). Androgens are also known to upregulate cAMP production in various tissues like testes, and prostate by either SHBG-receptors or an unknown GPCRs expressed by them(Heinlein and Chang, 2002; Rahman and Christian, 2007; Rosner et al., 1999; Shihan et al., 2014). Furthermore, an interaction of stimulatory G proteins and AR has been also well established in various prostate cancer cell lines(Kasbohm et al., 2005). All these reports directly or indirectly support our findings.

No published reports are available till date showing any known classical Androgen Response Elements (AREs) near the promoters of *FSHR, GNAS* and *RIC8B* genes in primates. Therefore, it seems that androgen may regulate these genes via a potential non classical pathway as reported in cultured rat Sc, for LDHA, EGR1 and CREB or CLDN11 mRNAs(Fix et al., 2004; Lui et al., 2007). Furthermore, androgen potentially targets multiple kinases which can induce different

418 sets of transcription factors in Sc (Walker, 2010). This event may become more feasible or
419 robust with pubertal maturation of testes as various transcription factors also gets upregulated
420 with Sc differentiation observed in rodents(Walker, 2003; Wood and Walker, 2009). It is critical
421 to note here that phosphorylated CREB in rat Sc is absolutely necessary for the survival of the
422 differentiating spermatocytes and T alone is sufficient to rescue the fertility in absence of FSH
423 by maintaining the minimal requirement of activated CREB (Scobey et al., 2001). Interestingly,
424 rat *FSHR* promoter can be activated by a cAMP response element like sequence (TGACTCA)
425 located at -561 to -568(Maguire et al., 1997; Monaco et al., 1995). Furthermore, a dramatic
426 decline in *FSHR* mRNA in AR knockout mice indirectly supports the probable role of T on
427 *FSHR* gene expression(Tan et al., 2005). An active CRE binding site has been recently reported
428 in human *RIC8B*gene promoter in neuroblasmotma cell line (Maureira et al., 2016).Taken
429 together it can be considered that T might be acting via non classical CREB mediated pathway to
430 augment genes like *FSHR*,*GNAS* and *RIC8B* mRNAs in pubertal primate Sc.

431 In our study, T did not alter the expression patterns of. i) inhibitory G proteins *GNAI* (Gαi)
432 subtypes 1, 2, 3 associated with the FSHR (Nikula et al., 1990), ii) regulator of G protein
433 signaling (RGS) *RGS-PX1*, which directly deactivates activated GNAS ($G\alpha_s$) thereby limiting
434 cAMP generation (Zheng et al., 2001), iii) *EPB41L2* which interferes with the GPCR-mediated
435 $G\alpha_s$ signaling necessary for activation of Adenylyl Cyclase (AC) (Goto et al., 2013) iv) *SCF* and
436 v) *GDNF* in both pubertal and infant Sc. However, in presence of T, FSH induced *SCF*, *GDNF*,
437 *ABP* and *Transferrin* mRNA expressions in pubertal Sc were augmented further. Such an effect
438 of T on FSH induced gene expression was not found in infant Sc. Additionally, supplementation
439 of FSH failed to show any substantial rise in the transcription of T induced *CLDN11*in pubertal
440 Sc. All these data together suggested that T signaling in pubertal Sc becomes favorable for FSH

action. It is essential to note here that, in infant primates, FSH action is restricted to proliferation of testicular Sc, whereas in pubertal testis, FSH shows pleiotropic effects from Sc proliferation to induction of robust Gc differentiation(Plant and Marshall, 2001). Our data suggested that, T mediated signaling may be responsible for the activation of such FSH action in pubertal Sc leading to gene expression necessary for robust Gc differentiation. This has been schematically explained by a cartoon in **Fig.6.**

Our data supported that pubertal Sc uniformly respond to T (an aromatizable androgen) and R1881 (a non-aromatizable androgen)for augmentation of *CLDN11* expression with or without FSH supplementation. However, it is essential to note that, significant Estradiol (E_2) production was observed when Testosterone (T) alone was supplemented in Sc culture. However, there was no discernible effect of FSH in further aromatization of T to E_2 ,consistent with our previous findings (Devi et al., 2006). Additionally, in this study, we have observed that there were no developmental difference in the ability of both infant and pubertal Sc in converting T to E_2. These observations further indicate that there is no significant impact of aromatization of T by Sc on developmentally regulated gene expression, studied here.

In summary, the finding suggested that T regulates the expression and activity of FSHR in pubertal Sc. To the best of our knowledge this is the first *in vitro* demonstration of androgen mediated augmentation of FSH signaling in primate Sc promoting gene expression for the pubertal onset of robust spermatogonial differentiation. Future studies are required to find out the relative contribution of the classical AR or SHBG-receptor/ other GPCR in this process (Heinlein and Chang, 2002; Kasbohm et al., 2005; Rahman and Christian, 2007; Rosner et al., 1999; Shihan et al., 2014). Additionally, a holistic functional approach is required for the better understanding of the regulation of primate Sc transcriptome during puberty. Furthermore, new

putative targets should be identified for evaluating synchronization of the crosstalk between androgen and FSH signaling network , in Sc which seems to be crucial for spermatogenesis.

7) Declaration of interest, Funding, Author's contributions, and Acknowledgements

Declaration of interest

All the authors declare that there is no conflict of interest

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Authors' Contributions

IB and SSM conceived the idea. IB and SSM participated in designing various components of this study. BSP, HS, PN and SSN were involved in generation and maintenance of GnRH driven juvenile monkeys. SSM and IB isolated, cultured and treated Sertoli cells from monkeys at various time points. IB and SB contributed to generation of the data. IB, BSP, HS and SSM analyzed the data.IB wrote the first draft of the manuscript. IB, HS and SSM prepared the final form of the manuscript.

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8) References

Abel, M.H., Baker, P.J., Charlton, H.M., Monteiro, A., Verhoeven, G., Gendt, K. De, Guillou, F., Shaughnessy, P.J.O., 2008. Spermatogenesis and Sertoli Cell Activity in Mice Lacking Sertoli Cell Receptors for Follicle-Stimulating Hormone and Androgen. Reactions 149, 3279 –3285. doi:10.1210/en.2008-0086

Abel, M.H., Wootton, A.N., Wilkins, V., Huhtaniemi, I., Knight, P.G., Charlton, H.M., 2000. The effect of a null mutation in the follicle-stimulating hormone receptor gene on mouse reproduction. Endocrinology 141, 1795–803.

Acosta, A.A., Khalifa, E., Oehninger, S., 1992. Pure human follicle stimulating hormone has a role in the treatment of severe male infertility by assisted reproduction: Norfolk's total experience. Hum. Reprod. 7, 1067–72.

Arslan, M., Weinbauer, G.F., Schlatt, S., Shahab, M., Nieschlag, E., 1993. FSH and testosterone,

alone or in combination, initiate testicular growth and increase the number of spermatogonia and Sertoli cells in a juvenile non-human primate (Macaca mulatta). J. Endocrinol. 136, 235–43.

Berensztein, E.B., Baquedano, M.S., Gonzalez, C.R., Saraco, N.I., Rodriguez, J., Ponzio, R., Rivarola, M.A., Belgorosky, A., 2006. Expression of aromatase, estrogen receptor alpha and beta, androgen receptor, and cytochrome P-450scc in the human early prepubertal testis. Pediatr. Res. 60, 740–4. doi:10.1203/01.pdr.0000246072.04663.bb

Bhattacharya, I., Basu, S., Sarda, K., Gautam, M., Nagarajan, P., Pradhan, B.S., Sarkar, H., Devi, Y.S., Majumdar, S.S., 2015. Low levels of Gαs and Ric8b in testicular sertoli cells may underlie restricted FSH action during infancy in primates. Endocrinology 156, 1143–55. doi:10.1210/en.2014-1746

Blok, L.J., Themmen, A.P., Peters, A.H., Trapman, J., Baarends, W.M., Hoogerbrugge, J.W., Grootegoed, J.A., 1992. Transcriptional regulation of androgen receptor gene expression in Sertoli cells and other cell types. Mol. Cell. Endocrinol. 88, 153–64.

Boukari, K., Meduri, G., Brailly-Tabard, S., Guibourdenche, J., Ciampi, M.L., Massin, N., Martinerie, L., Picard, J.-Y., Rey, R., Lombès, M., Young, J., 2009. Lack of androgen receptor expression in Sertoli cells accounts for the absence of anti-Mullerian hormone repression during early human testis development. J. Clin. Endocrinol. Metab. 94, 1818–25. doi:10.1210/jc.2008-1909

Bremner, W.J., Matsumoto, A.M., Sussman, A.M., Paulsen, C.A., 1981. Follicle-stimulating hormone and human spermatogenesis. J. Clin. Invest. 68, 1044–52. doi:10.1172/JCI110327

Bremner, W.J., Millar, M.R., Sharpe, R.M., Saunders, P.T.K., 1994. Immunohistochemical

localization of androgen receptors in the rat testis: Evidence for stage-dependent expression and regulation by androgens. Endocrinology 135, 1227–1234. doi:10.1210/endo.135.3.8070367

Burgués, S., Calderón, M.D., 1997. Subcutaneous self-administration of highly purified follicle stimulating hormone and human chorionic gonadotrophin for the treatment of male hypogonadotrophic hypogonadism. Spanish Collaborative Group on Male Hypogonadotropic Hypogonadism. Hum. Reprod. 12, 980–6.

Burris, A.S., Rodbard, H.W., Winters, S.J., Sherins, R.J., 1988. Gonadotropin therapy in men with isolated hypogonadotropic hypogonadism: the response to human chorionic gonadotropin is predicted by initial testicular size. J. Clin. Endocrinol. Metab. 66, 1144–51. doi:10.1210/jcem-66-6-1144

Buzzard, J.J., Wreford, N.G., Morrison, J.R., 2003. Thyroid hormone, retinoic acid, and testosterone suppress proliferation and induce markers of differentiation in cultured rat Sertoli cells. Endocrinology 144, 3722–3731. doi:10.1210/en.2003-0379

Chang, C., Lee, S.O., Wang, R.-S., Yeh, S., Chang, T.-M., 2013. Androgen receptor (AR) physiological roles in male and female reproductive systems: lessons learned from AR-knockout mice lacking AR in selective cells. Biol. Reprod. 89, 21. doi:10.1095/biolreprod.113.109132

Chapin, R.E., Phelps, J.L., Miller, B.E., Gray, T.J., 1987. Alkaline phosphatase histochemistry discriminates peritubular cells in primary rat testicular cell culture. J. Androl. 8, 155–61.

Chemes, H.E., Rey, R.A., Nistal, M., Regadera, J., Musse, M., González-Peramato, P., Serrano, A., 2008. Physiological androgen insensitivity of the fetal, neonatal, and early infantile

testis is explained by the ontogeny of the androgen receptor expression in Sertoli cells. J. Clin. Endocrinol. Metab. 93, 4408–12. doi:10.1210/jc.2008-0915

Cheng, J., Watkins, S.C., Walker, W.H., 2007. Testosterone activates mitogen-activated protein kinase via Src kinase and the epidermal growth factor receptor in sertoli cells. Endocrinology 148, 2066–2074. doi:10.1210/en.2006-1465

Devi, Y.S., Sarda, K., Stephen, B., Nagarajan, P., Majumdar, S.S., 2006. Follicle-stimulating hormone-independent functions of primate Sertoli cells: potential implications in the diagnosis and management of male infertility. J. Clin. Endocrinol. Metab. 91, 1062–1068. doi:10.1210/jc.2005-2072

Dierich, A., Sairam, M.R., Monaco, L., Fimia, G.M., Gansmuller, A., LeMeur, M., Sassone-Corsi, P., 1998. Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. Proc. Natl. Acad. Sci. U. S. A. 95, 13612–7.

Edelsztein, N.Y., Grinspon, R.P., Schteingart, H.F., Rey, R.A., 2016. Anti-Müllerian hormone as a marker of steroid and gonadotropin action in the testis of children and adolescents with disorders of the gonadal axis. Int. J. Pediatr. Endocrinol. 2016, 20. doi:10.1186/s13633-016-0038-2

Fix, C., Jordan, C.L., Cano, P., Walker, W.H., 2004. Testosterone activates mitogen-activated protein kinase and the cAMP response element binding protein transcription factor in Sertoli cells. Proc. Natl. Acad. Sci. U. S. A. 101, 10919–24. doi:10.1073/pnas.0404278101

Foresta, C., Bettella, A., Ferlin, A., Garolla, A., Rossato, M., 1998. Evidence for a stimulatory role of follicle-stimulating hormone on the spermatogonial population in adult males. Fertil.

Steril. 69, 636–642. doi:10.1016/S0015-0282(98)00008-9

Gorczynska, E., Handelsman, D.J., 1995. Androgens rapidly increase the cytosolic calcium concentration in Sertoli cells. Endocrinology 136, 2052–2059. doi:10.1210/endo.136.5.7720654

Goto, T., Chiba, A., Sukegawa, J., Yanagisawa, T., Saito, M., Nakahata, N., 2013. Suppression of adenylyl cyclase-mediated cAMP production by plasma membrane associated cytoskeletal protein 4.1G. Cell. Signal. 25, 690–697. doi:10.1016/j.cellsig.2012.11.020

Gromoll, J., Simoni, M., Nieschlag, E., 1996. An activating mutation of the follicle-stimulating hormone receptor autonomously sustains spermatogenesis in a hypophysectomized man. J. Clin. Endocrinol. Metab. 81, 1367–70. doi:10.1210/jcem.81.4.8636335

Hai, Y., Hou, J., Liu, Y., Liu, Y., Yang, H., Li, Z., He, Z., 2014. The roles and regulation of Sertoli cells in fate determinations of spermatogonial stem cells and spermatogenesis. Semin. Cell Dev. Biol. 29, 66–75. doi:10.1016/j.semcdb.2014.04.007

Heinlein, C.A., Chang, C., 2002. The Roles of Androgen Receptors and Androgen-Binding Proteins in Nongenomic Androgen Actions. Mol. Endocrinol. 16, 2181–2187. doi:10.1210/me.2002-0070

Holsberger, D.R., Jirawatnotai, S., Kiyokawa, H., Cooke, P.S., 2003. Thyroid hormone regulates the cell cycle inhibitor p27Kip1 in postnatal murine sertoli cells. Endocrinology 144, 3732–3738. doi:10.1210/en.2003-0389

Huhtaniemi, I., 2015. A short evolutionary history of FSH-stimulated spermatogenesis. Hormones (Athens). 14, 468–78. doi:10.14310/horm.2002.1632

Jonas, K.C., Oduwole, O.O., Peltoketo, H., Rulli, S.B., Huhtaniemi, I.T., 2014. Mouse models of

altered gonadotrophin action: insight into male reproductive disorders. Reproduction 148, R63–70. doi:10.1530/REP-14-0302

Kasbohm, E.A., Guo, R., Yowell, C.W., Bagchi, G., Kelly, P., Arora, P., Casey, P.J., Daaka, Y., 2005. Androgen receptor activation by Gs signaling in prostate cancer cells. J. Biol. Chem. 280, 11583–11589. doi:10.1074/jbc.M414423200

Kliesch, S., Behre, H.M., Nieschlag, E., 1995. Recombinant human follicle-stimulating hormone and human chorionic gonadotropin for induction of spermatogenesis in a hypogonadotropic male. Fertil. Steril. 63, 1326–8.

Klinefelter, G.R., Hall, P.F., Ewing, L.L., 1987. Effect of luteinizing hormone deprivation in situ on steroidogenesis of rat Leydig cells purified by a multistep procedure. Biol. Reprod. 36, 769–783. doi:10.1095/biolreprod36.3.769

Kumar, T.R., 2007. Mouse models for gonadotropins: a 15-year saga. Mol. Cell. Endocrinol. 260-262, 249–54. doi:10.1016/j.mce.2006.09.002

Kumar, T.R., Wang, Y., Lu, N., Matzuk, M.M., 1997. Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. Nat. Genet. 15, 201–204. doi:10.1038/ng0297-201

Kung, A.W., Zhong, Y.Y., Lam, K.S., Wang, C., 1994. Induction of spermatogenesis with gonadotrophins in Chinese men with hypogonadotrophic hypogonadism. Int. J. Androl. 17, 241–7.

Lee, B.C., Pineda, J.L., Spiliotis, B.E., Brown, T.J., Bercu, B.B., 1983. Male sexual development in the nonhuman primate. III. Sertoli cell culture and age-related differences. Biol. Reprod. 28, 1207–15.

ACCEPTED MANUSCRIPT

613 Lui, W.Y., Wong, E.W.P., Guan, Y., Lee, W.M., 2007. Dual transcriptional control of claudin-11
614 via an overlapping GATA/NF-Y motif: Positive regulation through the interaction of
615 GATA, NF-YA, and CREB and negative regulation through the interaction of Smad,
616 HDAC1, and mSin3A. J. Cell. Physiol. 211, 638–648. doi:10.1002/jcp.20970

617 Maguire, S.M., Tribley, W.A., Griswold, M.D., 1997. Follicle-Stimulating Hormone (FSH)
618 Regulates the Expression of FSH Receptor Messenger Ribonucleic Acid in Cultured Sertoli
619 Cells and in Hypophysectomized Rat Testis'. Biol. Reprod. 56, 1106–1111.
620 doi:10.1095/biolreprod56.5.1106

621 Majumdar, S.S., Sarda, K., Bhattacharya, I., Plant, T.M., 2012. Insufficient androgen and FSH
622 signaling may be responsible for the azoospermia of the infantile primate testes despite
623 exposure to an adult-like hormonal milieu. Hum. Reprod. 27, 2515–2525.
624 doi:10.1093/humrep/des184

625 Majumdar, S.S., Winters, S.J., Plant, T.M., 1998. Procedures for the isolation and culture of
626 Sertoli cells from the testes of infant, juvenile, and adult rhesus monkeys (Macaca mulatta).
627 Biol. Reprod. 58, 633–40.

628 Marshall, G.R., Jockenhövel, F., Lüdecke, D., Nieschlag, E., 1986. Maintenance of complete but
629 quantitatively reduced spermatogenesis in hypophysectomized monkeys by testosterone
630 alone. Acta Endocrinol. (Copenh). 113, 424–31.

631 Marshall, G.R., Wickings, E.J., Lüdecke, D.K., Nieschlag, E., 1983. Stimulation of
632 spermatogenesis in stalk-sectioned rhesus monkeys by testosterone alone. J. Clin.
633 Endocrinol. Metab. 57, 152–9. doi:10.1210/jcem-57-1-152

634 Marshall, G.R., Wickings, E.J., Nieschlag, E., 1984. Testosterone can initiate spermatogenesis in

635 an immature nonhuman primate, Macaca fascicularis. Endocrinology 114, 2228–33.
636 doi:10.1210/endo-114-6-2228

637 Marshall, G.R., Zorub, D.S., Plant, T.M., 1995. Follicle-stimulating hormone amplifies the
638 population of differentiated spermatogonia in the hypophysectomized testosterone-replaced
639 adult rhesus monkey (Macaca mulatta). Endocrinology 136, 3504–11.
640 doi:10.1210/endo.136.8.7628387

641 Matsumoto, A.M., Karpas, A.E., Bremner, W.J., 1986. Chronic human chorionic gonadotropin
642 administration in normal men: evidence that follicle-stimulating hormone is necessary for
643 the maintenance of quantitatively normal spermatogenesis in man. J. Clin. Endocrinol.
644 Metab. 62, 1184–92. doi:10.1210/jcem-62-6-1184

645 Matsumoto, A.M., Karpas, A.E., Paulsen, C.A., Bremner, W.J., 1983. Reinitiation of sperm
646 production in gonadotropin-suppressed normal men by administration of follicle-stimulating
647 hormone. J. Clin. Invest. 72, 1005–15.

648 Matsumoto, A.M., Paulsen, C.A., Bremner, W.J., 1984. Stimulation of Sperm Production by
649 Human Luteinizing Hormone in Gonadotropin-Suppressed Normal Men*. J. Clin.
650 Endocrinol. Metab. 59, 882–887. doi:10.1210/jcem-59-5-882

651 Matthiesson, K.L., McLachlan, R.I., O'Donnell, L., Frydenberg, M., Robertson, D.M., Stanton,
652 P.G., Meachem, S.J., 2006. The relative roles of follicle-stimulating hormone and
653 luteinizing hormone in maintaining spermatogonial maturation and spermiation in normal
654 men. J. Clin. Endocrinol. Metab. 91, 3962–9. doi:10.1210/jc.2006-1145

655 Maureira, A., Sánchez, R., Valenzuela, N., Torrejón, M., Hinrichs, M. V., Olate, J., Gutiérrez,
656 J.L., 2016. The CREB Transcription Factor Controls Transcriptional Activity of the Human

RIC8B Gene. J. Cell. Biochem. 1797–1805. doi:10.1002/jcb.25479

McKinnell, C., Saunders, P.T., Fraser, H.M., Kelnar, C.J., Kivlin, C., Morris, K.D., Sharpe, R.M., 2001. Comparison of androgen receptor and oestrogen receptor beta immunoexpression in the testes of the common marmoset (Callithrix jacchus) from birth to adulthood: low androgen receptor immunoexpression in Sertoli cells during the neonatal increase in testost. Reproduction 122, 419–29.

McLachlan, R.I., O'Donnell, L., Meachem, S.J., Stanton, P.G., de Kretser, D.M., Pratis, K., Robertson, D.M., 2002. Identification of specific sites of hormonal regulation in spermatogenesis in rats, monkeys, and man. Recent Prog. Horm. Res. 57, 149–79.

Monaco, L., Foulkes, N.S., Sassone-Corsi, P., 1995. Pituitary follicle-stimulating hormone (FSH) induces CREM gene expression in Sertoli cells: involvement in long-term desensitization of the FSH receptor. Proc. Natl. Acad. Sci. U. S. A. 92, 10673–7. doi:10.1073/pnas.92.23.10673

Nieschlag, E., Simoni, M., Gromoll, J., Weinbauer, G.F., 1999. Role of FSH in the regulation of spermatogenesis: clinical aspects. Clin. Endocrinol. (Oxf). 51, 139–46.

Nikula, H., Vihko, K., Huhtaniemi, I., 1990. Protein kinase C and Gi-protein mediated modulation of cAMP production in different stages of the rat seminiferous epithelium. Mol. Cell. Endocrinol. 70, 247–253. doi:10.1016/0303-7207(90)90215-T

O'Shaughnessy, P.J., Monteiro, A., Abel, M., 2012. Testicular Development in Mice Lacking Receptors for Follicle Stimulating Hormone and Androgen. PLoS One 7, e35136. doi:10.1371/journal.pone.0035136

Oduwole, O.O., Peltoketo, H., Poliandri, A., Vengadabady, L., Chrusciel, M., Doroszko, M.,

679 Samanta, L., Owen, L., Keevil, B., Rahman, N.A., Huhtaniemi, I.T., 2018. Constitutively
680 active follicle-stimulating hormone receptor enables androgen-independent
681 spermatogenesis. J. Clin. Invest. 128, 1787–1792. doi:10.1172/JCI96794

682 Phillip, M., Arbelle, J.E., Segev, Y., Parvari, R., 1998. Male Hypogonadism Due to a Mutation
683 in the Gene for the β-Subunit of Follicle-Stimulating Hormone. N. Engl. J. Med. 338, 1729–
684 1732. doi:10.1056/NEJM199806113382404

685 Plant, T.M., Marshall, G.R., 2001. The functional significance of FSH in spermatogenesis and
686 the control of its secretion in male primates. Endocr. Rev. doi:10.1210/edrv.22.6.0446

687 Rahman, F., Christian, H.C., 2007. Non-classical actions of testosterone: an update. Trends
688 Endocrinol. Metab. doi:10.1016/j.tem.2007.09.004

689 Ramaswamy, S., Marshall, G.R., Pohl, C.R., Friedman, R.L., Plant, T.M., 2003. Inhibitory and
690 stimulatory regulation of testicular inhibin B secretion by luteinizing hormone and follicle-
691 stimulating hormone, respectively, in the rhesus monkey (Macaca mulatta). Endocrinology
692 144, 1175–1185. doi:10.1210/en.2002-221078

693 Ramaswamy, S., Plant, T.M., Marshall, G.R., 2000. Pulsatile stimulation with recombinant
694 single chain human luteinizing hormone elicits precocious sertoli cell proliferation in the
695 juvenile male rhesus monkey (Macaca mulatta). Biol. Reprod. 63, 82–8.

696 Rey, R.A., 2014. Mini-puberty and true puberty: Differences in testicular function. Ann.
697 Endocrinol. (Paris). 75, 58–63. doi:10.1016/j.ando.2014.03.001

698 Rey, R.A., Musse, M., Venara, M., Chemes, H.E., 2009. Ontogeny of the androgen receptor
699 expression in the fetal and postnatal testis: its relevance on Sertoli cell maturation and the
700 onset of adult spermatogenesis. Microsc. Res. Tech. 72, 787–95. doi:10.1002/jemt.20754

701 Rosner, W., Hryb, D.J., Khan, M.S., Nakhla, a M., Romas, N. a, 1999. Androgen and estrogen
702 signaling at the cell membrane via G-proteins and cyclic adenosine monophosphate.
703 Steroids 64, 100–6. doi:Doi 10.1016/S0039-128x(98)00108-1

704 Ruwanpura, S.M., McLachlan, R.I., Meachem, S.J., 2010. Hormonal regulation of male germ
705 cell development. J. Endocrinol. 205, 117–131. doi:10.1677/JOE-10-0025

706 Schlatt, S., Arslan, M., Weinbauer, G.F., Behre, H.M., Nieschlag, E., 1995. Endocrine control of
707 testicular somatic and premeiotic germ cell development in the immature testis of the
708 primate Macaca mulatta. Eur. J. Endocrinol. 133, 235–47.

709 Scobey, M.J., Bertera, S., Somers, J.P., Watkins, S.C., Zeleznik, A.J., Walker, W.H., 2001.
710 Delivery of a cyclic adenosine 3′,5′-monophosphate response element-binding protein
711 (CREB) mutant to seminiferous tubules results in impaired spermatogenesis. Endocrinology
712 142, 948–954. doi:10.1210/endo.142.2.7948

713 Sen, A., Prizant, H., Light, A., Biswas, A., Hayes, E., Lee, H.-J., Barad, D., Gleicher, N.,
714 Hammes, S.R., 2014. Androgens regulate ovarian follicular development by increasing
715 follicle stimulating hormone receptor and microRNA-125b expression. Proc. Natl. Acad.
716 Sci. 111, 3008–3013. doi:10.1073/pnas.1318978111

717 Shan, L.X., Bardin, C.W., Hardy, M.P., 1997. Immunohistochemical analysis of androgen effects
718 on androgen receptor expression in developing leydig and Sertoli cells. Endocrinology 138,
719 1259–1266. doi:10.1210/endo.138.3.4973

720 Sharpe, R., 1994. Regulation of spermatogenesis., in: Knobil, E., Neill, J. (Eds.), The Physiology
721 of Reproduction. Raven Press, New York, pp. 1363–1434.

722 Sharpe, R.M., McKinnell, C., Kivlin, C., Fisher, J.S., 2003. Proliferation and functional

723 maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood.
724 Reproduction 125, 769–84.

725 Shihan, M., Bulldan, A., Scheiner-Bobis, G., 2014. Non-classical testosterone signaling is
726 mediated by a G-protein-coupled receptor interacting with Gnα11. Biochim. Biophys. Acta
727 1843, 1172–81. doi:10.1016/j.bbamcr.2014.03.002

728 Shupe, J., Cheng, J., Puri, P., Kostereva, N., Walker, W.H., 2011. Regulation of Sertoli-Germ
729 Cell Adhesion and Sperm Release by FSH and Nonclassical Testosterone Signaling. Mol.
730 Endocrinol. 25, 238–252. doi:10.1210/me.2010-0030

731 Simoni, M., Casarini, L., 2014. MECHANISMS IN ENDOCRINOLOGY: Genetics of FSH
732 action: a 2014-and-beyond view. Eur. J. Endocrinol. 170, R91–R107. doi:10.1530/EJE-13-
733 0624

734 Simorangkir, D.R., Ramaswamy, S., Marshall, G.R., Pohl, C.R., Plant, T.M., 2009. A selective
735 monotropic elevation of FSH, but not that of LH, amplifies the proliferation and
736 differentiation of spermatogonia in the adult rhesus monkey (Macaca mulatta). Hum.
737 Reprod. 24, 1584–1595. doi:10.1093/humrep/dep052

738 Smith LB, W.W., 2015. Hormonal signaling in the testis, in: Plant TM, Z.A. (Ed.), Knobil and
739 Neill's Physiology of Reproduction. Waltham, Massachusetts, USA: Academic Press, pp.
740 635–675.

741 Steger, K., Rey, R., Louis, F., Kliesch, S., Behre, H.M., Nieschlag, E., Hoepffner, W., Bailey, D.,
742 Marks, A., Bergmann, M., 1999. Reversion of the differentiated phenotype and maturation
743 block in Sertoli cells in pathological human. Hum. Reprod. 14, 136–143.
744 doi:10.1093/humrep/14.1.136

ACCEPTED MANUSCRIPT

745 Steinberger, E., Root, A., Ficher, M., Smith, K.D., 1973. The Role of Androgens in the Initiation
746 of Spermatogenesis in Man. J. Clin. Endocrinol. Metab. 37, 746–751. doi:10.1210/jcem-37-
747 5-746

748 Tan, K.A.L., De Gendt, K., Atanassova, N., Walker, M., Sharpe, R.M., Saunders, P.T.K.,
749 Denolet, E., Verhoeven, G., 2005. The role of androgens in sertoli cell proliferation and
750 functional maturation: Studies in mice with total or sertoli cell-selective ablation of the
751 androgen receptor. Endocrinology 146, 2674–2683. doi:10.1210/en.2004-1630

752 Tapanainen, J.S., Aittomäki, K., Min, J., Vaskivuo, T., Huhtaniemi, I.T., 1997. Men homozygous
753 for an inactivating mutation of the follicle-stimulating hormone (FSH) receptor gene present
754 variable suppression of spermatogenesis and fertility. Nat. Genet. 15, 205–6.
755 doi:10.1038/ng0297-205

756 Toocheck, C., Clister, T., Shupe, J., Crum, C., Ravindranathan, P., Lee, T.-K., Ahn, J.-M., Raj,
757 G. V., Sukhwani, M., Orwig, K.E., Walker, W.H., 2016. Mouse Spermatogenesis Requires
758 Classical and Nonclassical Testosterone Signaling1. Biol. Reprod. 94.
759 doi:10.1095/biolreprod.115.132068

760 Van Alphen, M.M.A., Van De Kant, H.J.G., De ROOIJ, D.G., 1988. Follicle-Stimulating
761 Hormone Stimulates Spermatogenesis in the Adult Monkey*. Endocrinology 123, 1449–
762 1455. doi:10.1210/endo-123-3-1449

763 Verhoeven, G., Cailleau, J., 1988. Follicle-stimulating hormone and androgens increase the
764 concentration of the androgen receptor in sertoli cells. Endocrinology 122, 1541–1550.
765 doi:10.1210/endo-122-4-1541

766 Verhoeven, G., Willems, A., Denolet, E., Swinnen, J. V., De Gendt, K., 2010. Androgens and

767 spermatogenesis: lessons from transgenic mouse models. Philos. Trans. R. Soc. B Biol. Sci.
768 365, 1537–1556. doi:10.1098/rstb.2009.0117

769 Viswanathan, P., Wood, M.A., Walker, W.H., 2009. Follicle-stimulating hormone (FSH)
770 transiently blocks FSH receptor transcription by increasing inhibitor of deoxyribonucleic
771 acid binding/differentiation-2 and decreasing upstream stimulatory factor expression in rat
772 Sertoli cells. Endocrinology 150, 3783–91. doi:10.1210/en.2008-1261

773 Walker, W.H., 2010. Non-classical actions of testosterone and spermatogenesis. Philos. Trans. R.
774 Soc. B Biol. Sci. doi:10.1098/rstb.2009.0258

775 Walker, W.H., 2003. Molecular mechanisms controlling Sertoli cell proliferation and
776 differentiation. Endocrinology 144, 3719–21. doi:10.1210/en.2003-0765

777 Walker, W.H., Cheng, J., 2005. FSH and testosterone signaling in Sertoli cells. Reproduction
778 130, 15–28. doi:10.1530/rep.1.00358

779 Weinbauer, G.F., Behre, H.M., Fingscheidt, U., Nieschlag, E., 1991. Human follicle-stimulating
780 hormone exerts a stimulatory effect on spermatogenesis, testicular size, and serum inhibin
781 levels in the gonadotropin-releasing hormone antagonist-treated nonhuman primate
782 (Macaca fascicularis). Endocrinology 129, 1831–9. doi:10.1210/endo-129-4-1831

783 Weinbauer, G.F., Göckeler, E., Nieschlag, E., 1988. Testosterone prevents complete suppression
784 of spermatogenesis in the gonadotropin-releasing hormone antagonist-treated nonhuman
785 primate (Macaca fascicularis). J. Clin. Endocrinol. Metab. 67, 284–90. doi:10.1210/jcem-
786 67-2-284

787 Wood, M.A., Walker, W.H., 2009. USF1/2 transcription factor DNA-binding activity is induced
788 during rat Sertoli cell differentiation. Biol. Reprod. 80, 24–33.

ACCEPTED MANUSCRIPT

789 doi:10.1095/biolreprod.108.070037

790 Young, J., Rey, R., Couzinet, B., Chanson, P., Josso, N., Schaison, G., Supe, E.N., 1999.
791 Antimüllerian Hormone in Patients with hypogonadotropic hypogonadism. J. Clin.
792 Endocrinol. Metab. 84, 2696–9. doi:10.1210/jcem.84.8.5972

793 Zheng, B., Ma, Y.C., Ostrom, R.S., Lavoie, C., Gill, G.N., Insel, P. a, Huang, X.Y., Farquhar,
794 M.G., 2001. RGS-PX1, a GAP for GalphaS and sorting nexin in vesicular trafficking.
795 Science (80-.). 294, 1939–42. doi:10.1126/science.1064757

796 Zheng, J., Mao, J., Cui, M., Liu, Z., Wang, X., Xiong, S., Nie, M., Wu, X., 2017. Novel FSHβ
797 mutation in a male patient with isolated FSH deficiency and infertility. Eur. J. Med. Genet.
798 60, 335–339. doi:10.1016/j.ejmg.2017.04.004

799 Zhu, L.J., Hardy, M.P., Inigo, I. V, Huhtaniemi, I., Bardin, C.W., Moo-Young, A.J., 2000.
800 Effects of androgen on androgen receptor expression in rat testicular and epididymal cells: a
801 quantitative immunohistochemical study. Biol. Reprod. 63, 368–76.

802 **Figure Legends**

803 **Figure 1. Androgen mediated gene expression in pubertal and infant monkey Sc.**

804 Shows relative quantities of mRNA expressions of *Inhibinβ-B*(*INHBB*) (A1), *AMH* (B1), *CK18*
805 (C1) and *CLDN11* (D1) by T or R1881 in pubertal Sc and *INHBB* (A2), *AMH* (B2), *CK18* (C2)
806 and *CLDN11* (D2) by T only in infant Sc at 24hr. In all cases, the mean±SEM of at least three
807 independent experiments for each age group was analyzed. Statistical significance ($p < 0.05$) by
808 one way ANOVA followed by Dunnet's post-test (basal was taken as control for each age group)

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809 is indicated by asterisk (*p<0.05, **p<0.01, and ***p<0.001). C: Control, T: Testosterone,
810 R1881: Synthetic Androgen.

811 **Figure 2. T mediated augmentation of FSH induced gene expression associated with FSH**
812 **signaling in pubertal and infant monkey Sc.**

813 Shows relative quantities of mRNA expressions of *FSHR* (A), *GNAS* (B), *RIC8B* (C) in pubertal
814 Sc and *FSHR* (D), *GNAS* (E), *RIC8B* (F) in infant Sc at 24hr. In all cases, the Mean±SEM of at
815 least three independent experiments for each age group was analyzed and statistical significance
816 was estimated by ANOVA followed by Dunnet's post-test (basal was taken as control for each
817 age group). Significant difference are marked with asterisk (*p<0.05, **p<0.01, and
818 ***p<0.001). C: Control, F: FSH, T: Testosterone and FT: FSH and Testosterone in
819 combination.

820

821 **Figure3. Androgen mediated augmentation of gene expression associated with FSH**
822 **signaling in pubertal and infant monkey Sc.**

823 Shows relative quantities of mRNA expressions of *FSHR* (A1), *GNAS* (B1), *RIC8B* (C1) by
824 either T or R1881 in pubertal Sc and *FSHR* (A2), *GNAS* (B2), *RIC8B* (D2) by T only in infant Sc
825 at 24hr. In all cases, the Mean±SEM of at least three independent experiments for each age group
826 was analyzed and statistical significance was estimated by ANOVA followed by Dunnet's post-
827 test (basal was taken as control for each age group). Significant difference are marked with
828 asterisk (*p<0.05, **p<0.01 and ***p<0.001). C: Control, T: Testosterone, R1881: Synthetic
829 Androgen.

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Figure 4. Hormone induced cAMP production by pubertal and infant monkey Sc at 1/2hr to 24hr.

The level of cAMP production (pmole/ml/million Sc in Y axis) at 24hr in pubertal Sc (**A**), ½ hr in pubertal Sc (**B**); and½ hr in infant Sc(**C**), 24hr in infant Sc (**D**). In all cases, the Mean±SEM of at least three independent experiments for each age group was analyzed and statistical significance was estimated by ANOVA followed by Dunnet's post-test (basal was taken as control for each age group) and comparison between two groups are done by Student t-test. Significant difference are marked with asterisk (*p<0.05, **p<0.01 and ***p<0.001). C: Control, R1881: Synthetic Androgen, T: Testosterone, F: FSH, FT: FSH and Testosterone in combination.

Figure 5. FSH and T (either alone or in combination) mediated gene expression in pubertal and infant monkey Sc.

Shows relative quantities of mRNA expressions of *SCF* (**A&E**), *GDNF*(**B& F**), *ABP*(**C& G**) and *Transferrin* (*TF*) (**D&H**) in pubertal(Pub) and infant (Inf) Sc at 24 hr of treatment with FSH (F), Testosterone (T) and both FSH and Testosterone in combination (FT)with IBMX (0.1mM). Each bar represents the Mean±SEM from at least three individual monkeys (N>3). Statistical significance was estimated by ANOVA followed by Dunnett's post-test using Vehicle or FSH treated group as control. Asterisk indicates significant differences (*p<0.05, **p<0.01 and ***p<0.001).

Fig: 6.A cartoon showing T mediated augmentation in FSH signaling leading to robust gene expression in pubertal Sc but not in infant Sc.

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852 **Supplemental Figure S1.**

853 **Hematoxylin staining and OilRedO staining of cultured Sertoli cells (Sc) isolated from**
854 **infant (A&C) and pubertal (B&D) monkey testes.**

855

856 **Supplemental Figure S2.**

857 **FSH and T mediated gene expression in pubertal and infant Sc.**

858 Shows relative quantities of mRNA expressions of *INHBB* (**A1&A2**), *AMH* (**B1&B2**) *CK18*
859 (**C1&C2**) and *CLDN11* (**D1&D2**) in pubertal (Pub) and infant (Inf) Sc at 24 hr of treatment with
860 FSH (F), and both FSH and Testosterone in combination (FT). Each bar represents the
861 Mean±SEM from at least three individual monkeys (N>3). Statistical significance was estimated
862 by ANOVA followed by Dunnett's post-test. Asterisk indicates significant differences (*$p<0.05$,
863 **$p<0.01$).

864

865 **Supplemental Figure S3.**

866 **T mediated AR mRNA expression in pubertal and infant Sc.**

867 Shows relative quantities of *AR* mRNAs in pubertal (A) and infant (B) Sc at 24 hr of treatment
868 with vehicle alone (Control= C) and vehicle with FSH (F) and/or Testosterone (T) alone or in
869 combination (FT). Each bar represents the Mean±SEM from at least three individual monkeys
870 (N>3). Statistical significance was estimated by ANOVA followed by Dunnett's post-test.

871

872

Supplemental Figure S4.

T mediated gene expression in pubertal and infant Sc.

Shows relative quantities of mRNAs of *GNAI1* (**A**), *GNAI2* **B**), *GNAI3* (**C**),*RGS-PX1* (**D**) and *EPB41L2* (**E**) in pubertal (Pub) and infant (Inf) Sc at 24 hr of treatment with vehicle alone (Control= C) and vehicle with Testosterone (T). Each bar represents the Mean±SEM from at least three individual monkeys (N>3). Statistical significance was estimated by ANOVA followed by Dunnett's post-test.

Supplemental Figure S5.

Production of E_2 by pubertal and infant Sc upon treatment of FSH and T either alone or in combination at 24hr.

Level of E_2 production (pg X 10^2/ml/million Sc in Y axis) in pubertal Sc (**A**) and infant Sc (**B**). In all cases, the Mean±SEM of at least three independent experiments for each age group was analyzed and statistical significance was estimated by ANOVA followed by Tukeys Multiple Comparison Test (N=3). C: Control, T: Testosterone, F: FSH, FT: FSH and Testosterone in combination.

Supplemental Figure S6.

Hormone mediated CLDN11 expression in pubertal Sc.

Shows relative quantities of *CLDN11* mRNAs in pubertal Sc at 24 hr of treatment with vehicle alone (Control= C) and vehicle with FSH and/or Androgen [Testosterone (T) or R1881]. Each

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bar represents the Mean±SEM from at least three individual monkeys (N>3). Statistical significance was estimated by ANOVA followed by Dunnett's post-test.

Table S1.
List of primers used in Q-RT PCR

Table S2.
Plasma levels of Testosterone (T) in infant ant and GnRH treated juvenile monkeys

Table S3.
P values calculated by statistical method (student t-test) between different groups as shown in Figure- 4 A-D.

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Table -1: Overview of experimental flowchart of Sc cultures and treatments

Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Castration of monkeys and Sertoli Cell (Sc) were isolated &cultured in DMEM/F12 HAM with 1% fetal calf serum (FCS) for 24hr.	FCS media were replaced by 1% Serum replacement growth factors (GF)& cultures were maintained for next 24hr.	Old GF media were replaced by fresh GF media& cultures were maintained for next 24hr.	Hypotonic shock were given by Tris-HCL (20mM , pH=7.4) to remove Germ Cells (Gc) &cells were washed twice with media alone and cultures were maintained in GF media for next 24hr.	Cytochemicalevaluation was done to evaluate the purity of Sc cultures. For *in vitro*treatments, cells were exposed to i) vehicle only (with GF media with 0.1 mM IBMX) ii) vehicle with T or synthetic androgen R1881 (100 nM) , iii) vehicle with rmFSH (5ng/ml) and iv) vehicle with both T and FSH for next 1/2 hr (for cAMP only), and 24hr (for cAMP, E_2 and/or mRNA expression).	*In vitro* treatments were terminated&hormone treated and untreated cells were saved in Trizol reagent for RNA isolation&Sc exposed media were used to evaluate cAMP& E_2 production.

Fig. 1: Androgen mediated gene expression in pubertal and infant monkey Sc.

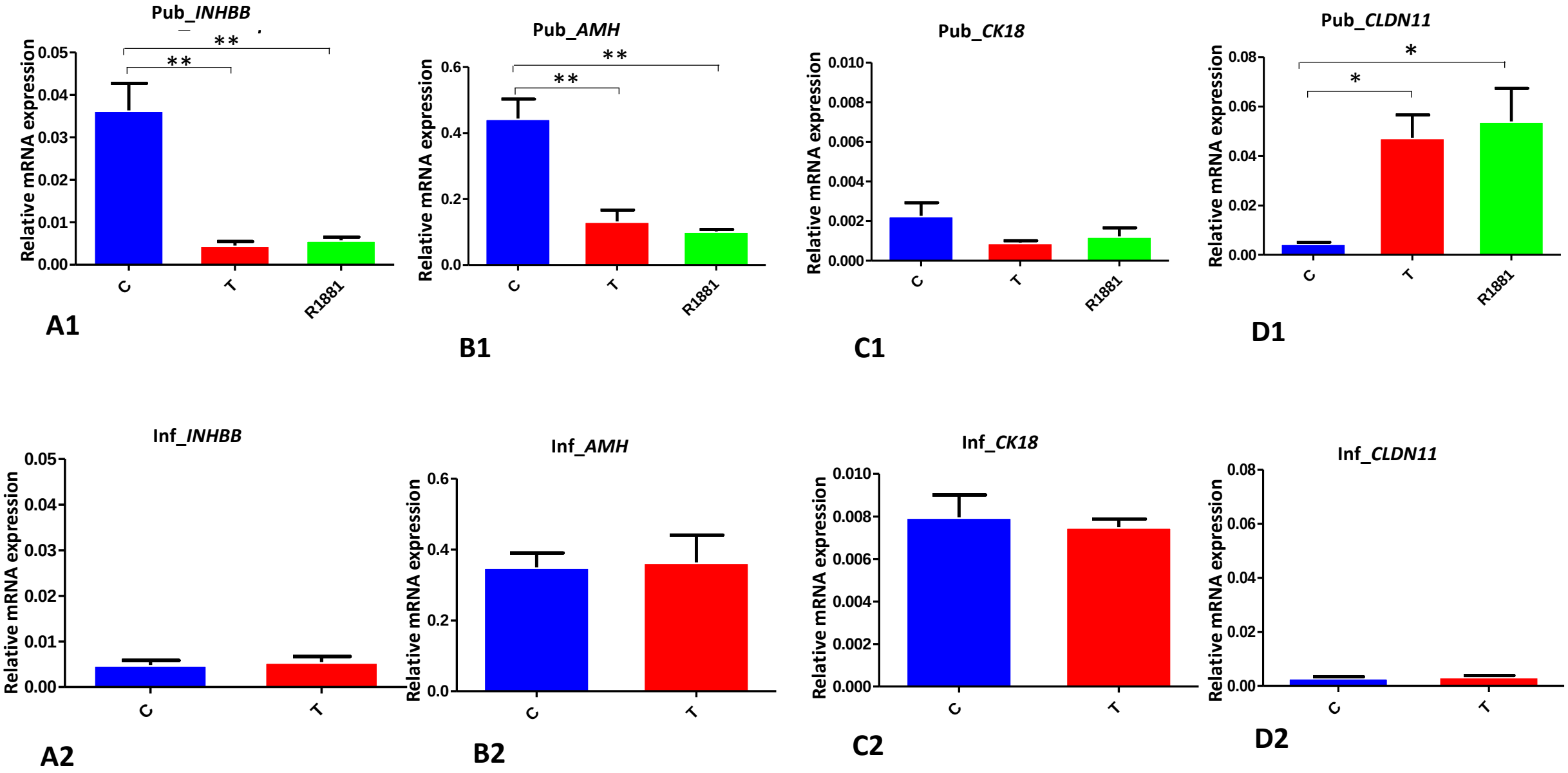


Fig. 2: T mediated augmentation of FSH induced gene expression associated with FSH signaling in pubertal and infant monkey Sc.

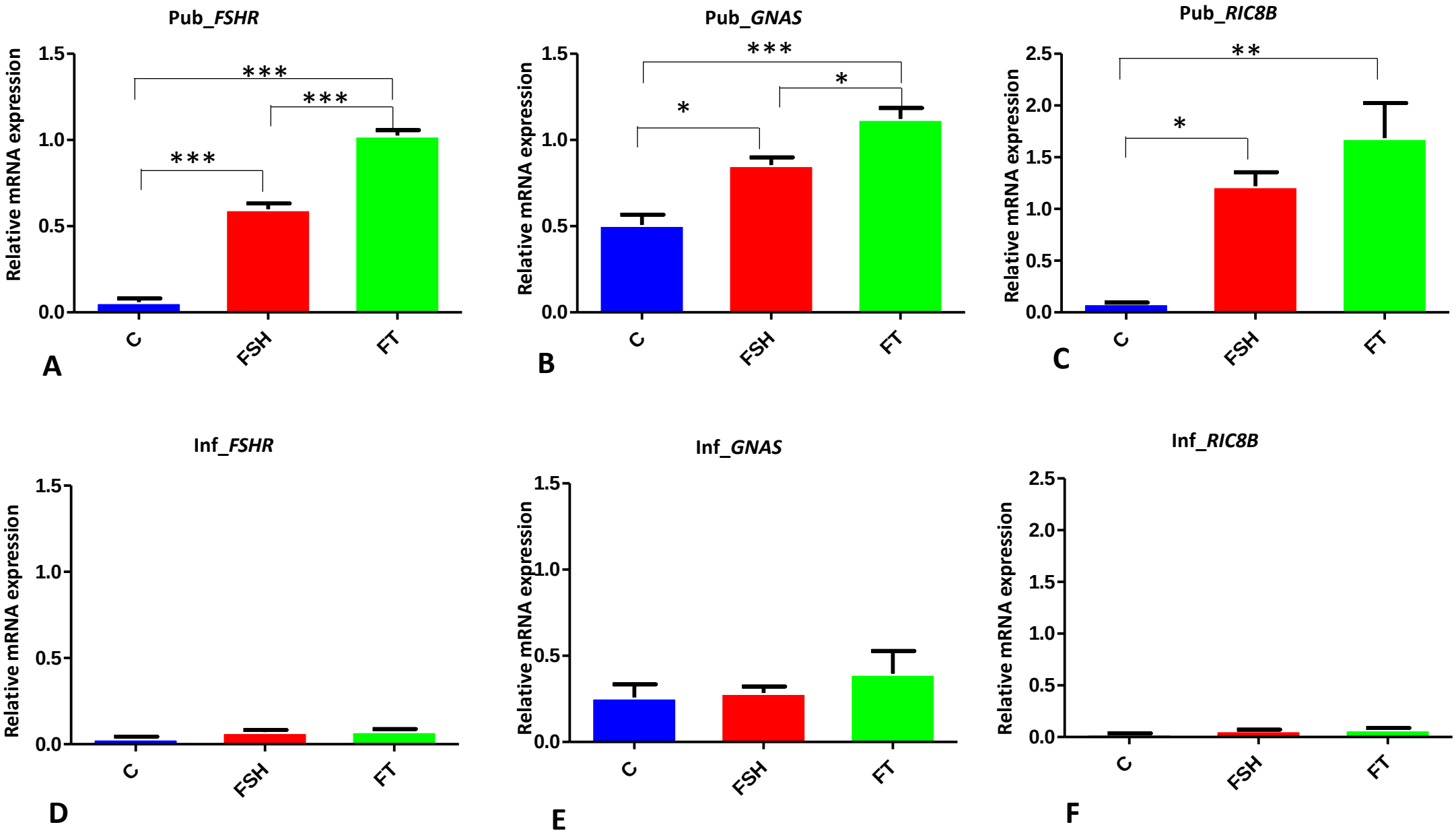
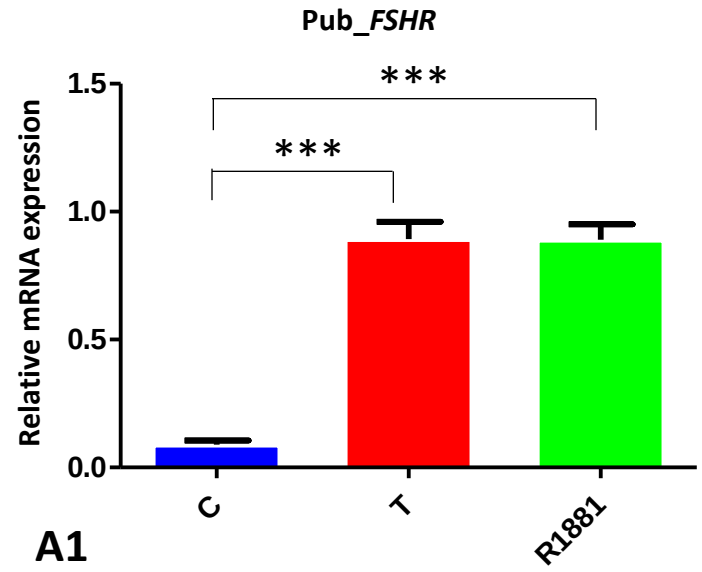
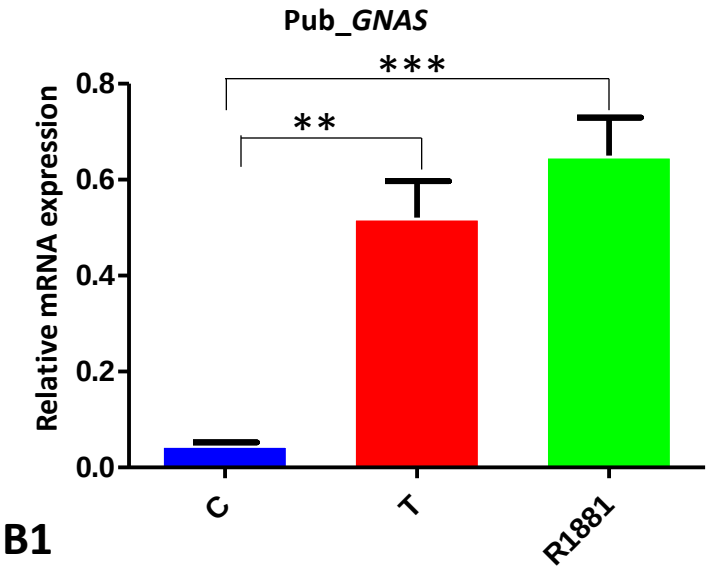


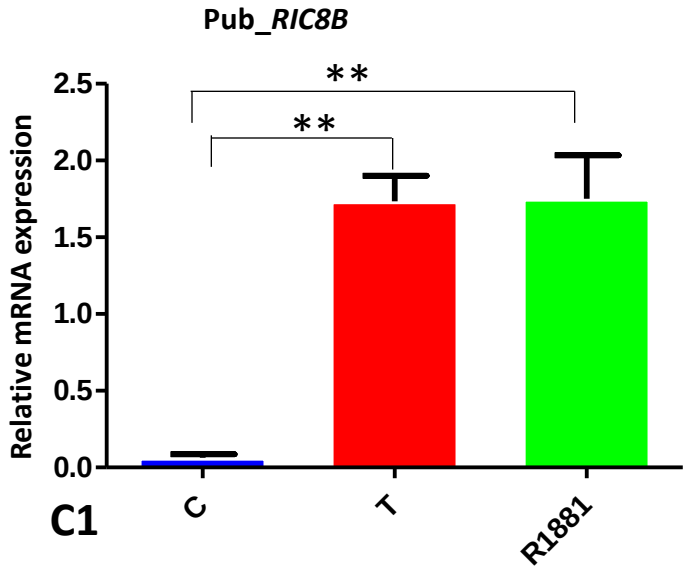
Fig. 3: Androgen mediated augmentation of gene expression associated with FSH signaling by pubertal and infant monkey Sc.



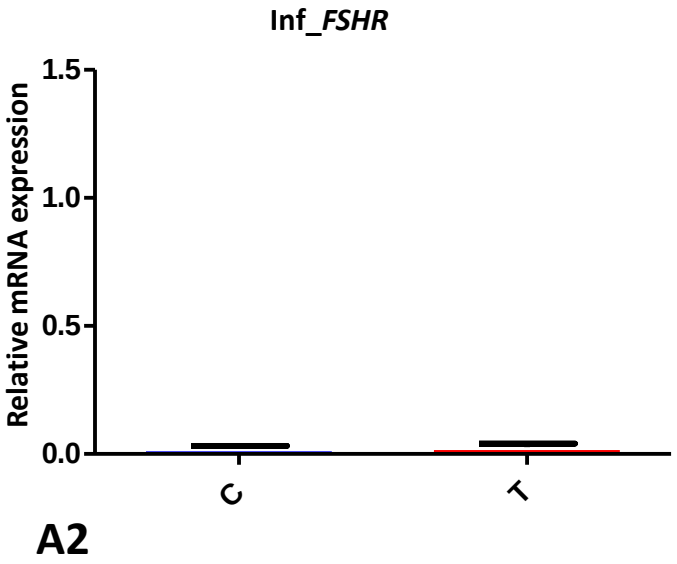
A1



B1



C1



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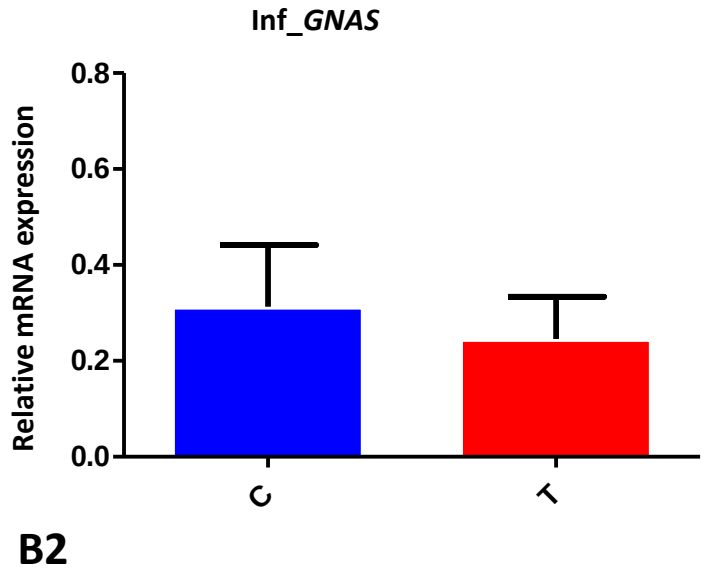
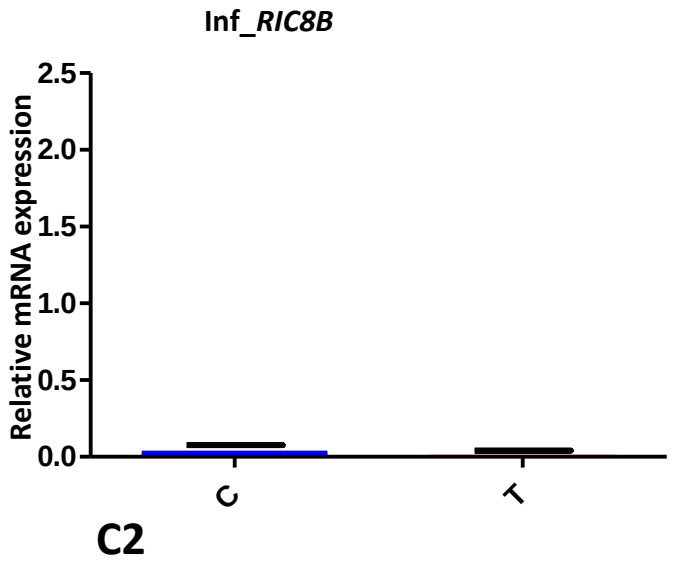


B2



C2

Fig. 4: Hormone induced cAMP production by pubertal and infant monkey Sc at 1/2hr to 24hr.

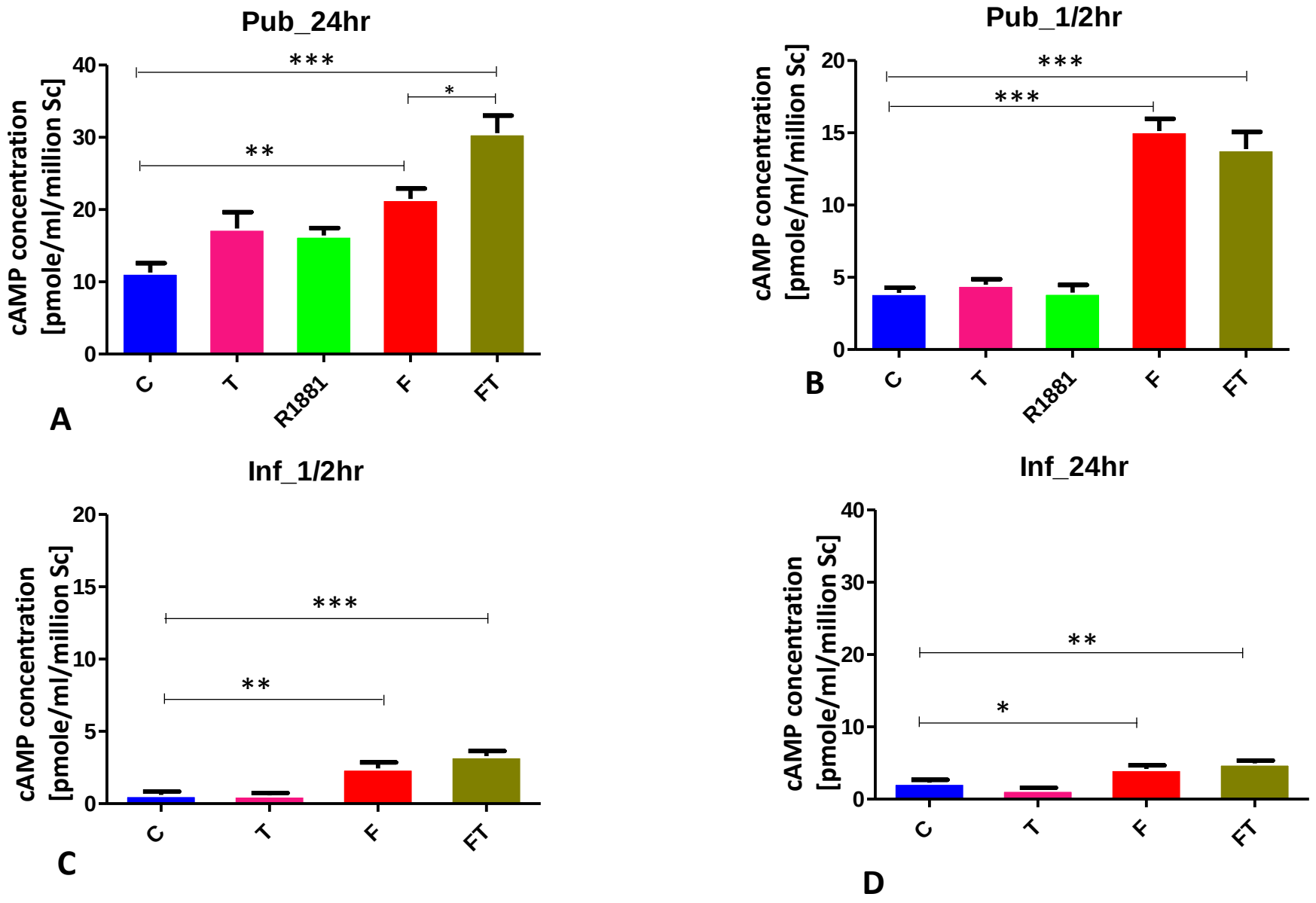


Fig. 5: FSH and T (either alone or in combination) mediated gene expression in pubertal and infant monkey Sc.

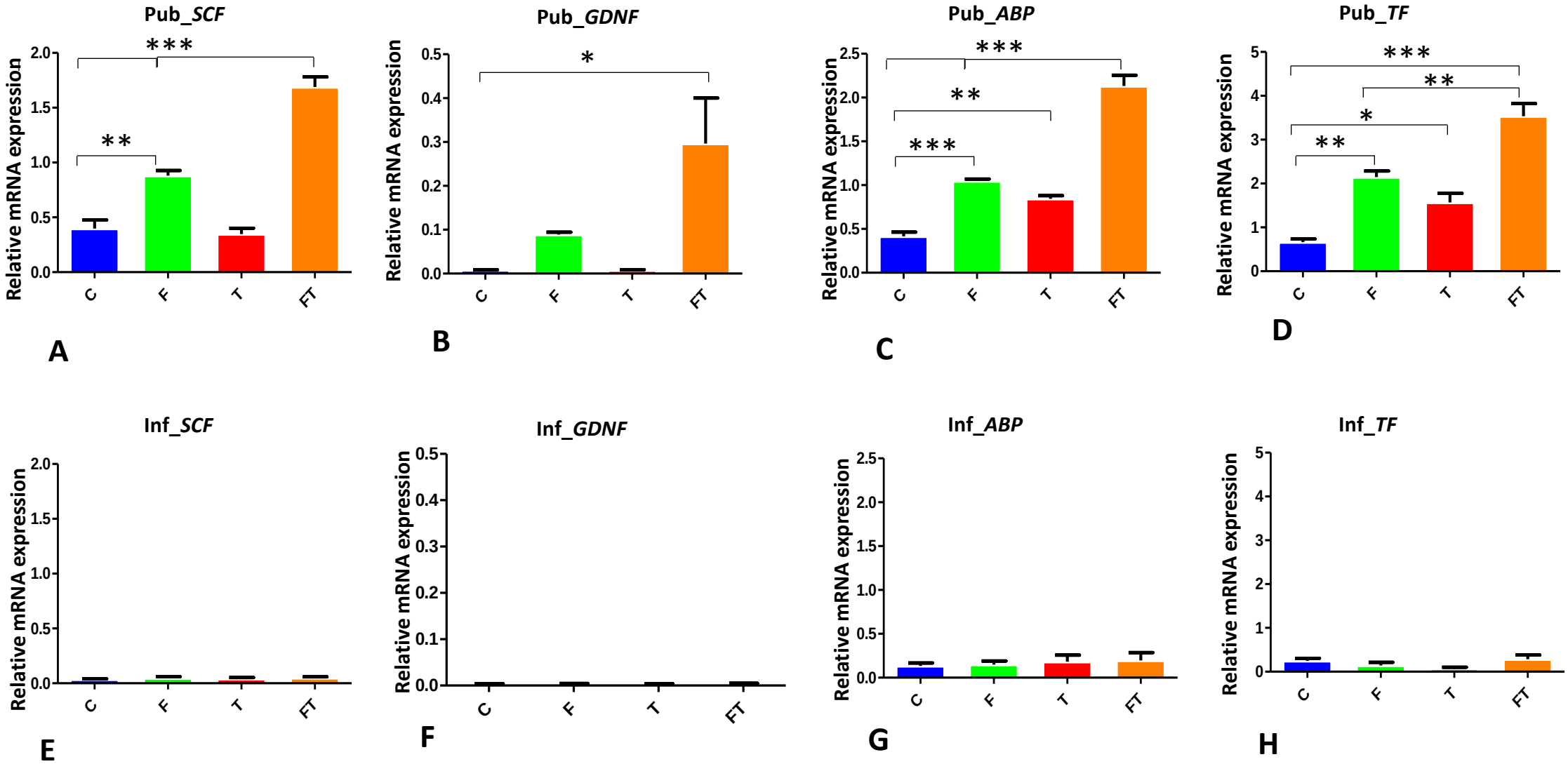
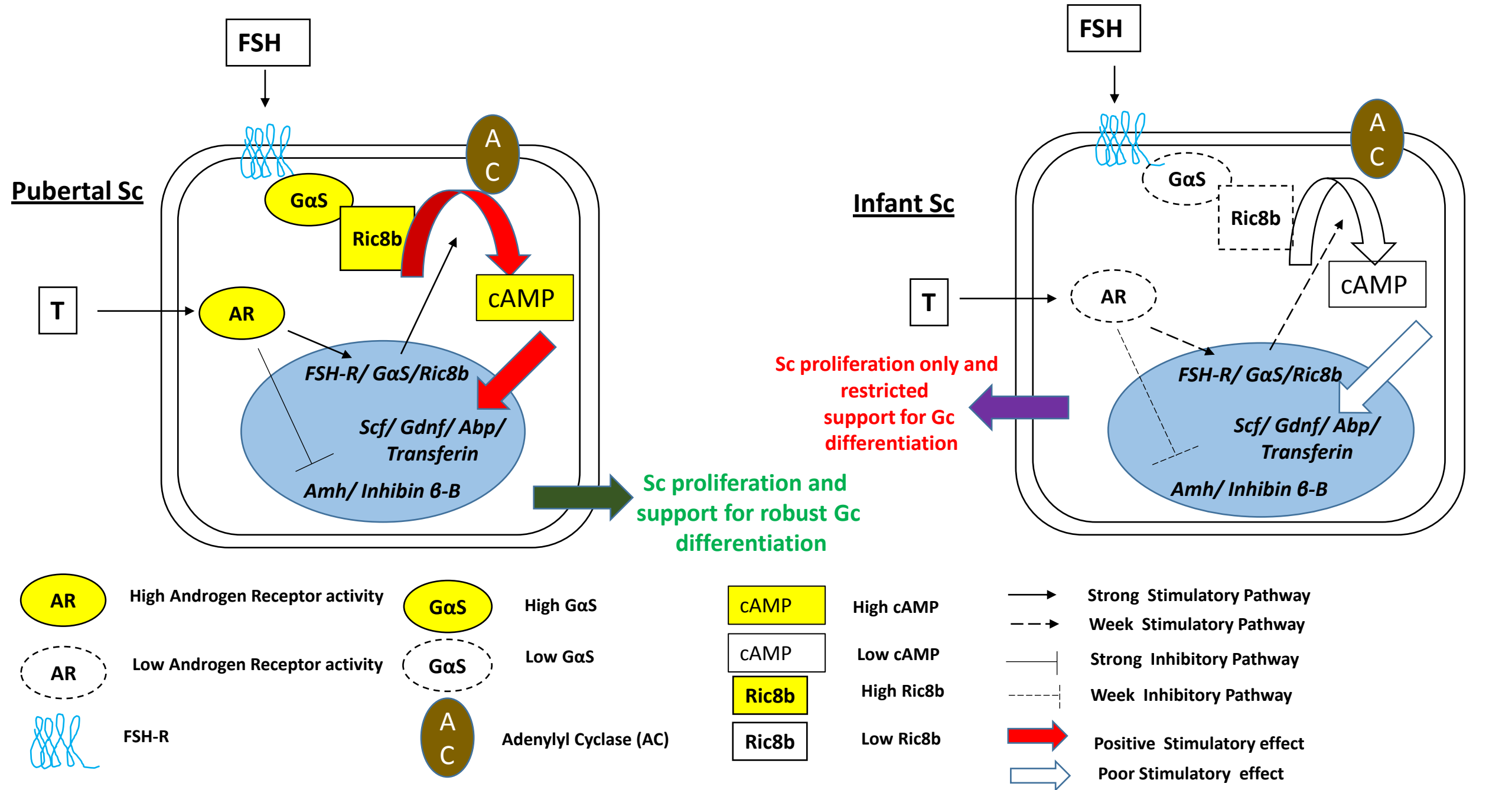


Fig. 6: T mediated augmentation in FSH signaling leading to robust gene expression in pubertal Sc but not in Infant Sc



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Highlights

- Pubertal Primate Sertoli cells (Sc) show androgen mediated down-regulation of AMH and Inhibin-βB expressions.

- Unlike Infant Sc, Androgen upregulates FSH-Receptor, Gαs subunit and Ric8b only in pubertal Sc.

- Testosterone (T) gets aromatized to Estrogen (E_2) uniformly in both infant and pubertal Sc independent of FSH stimulation.

- T augments FSH induced SCF, GDNF, ABP and Transferrin expression only in pubertal Sc.

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