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Stem Cell Reports
Article

OPEN ACCESS

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https://doi.org/10.1016/j.stemcr.2018.08.018

SUMMARY

Several studies have reported an association between the farnesoid X receptor alpha (FXRα) and estrogenic signaling pathways. Fxrα could thus be involved in the reprotoxic effects of endocrine disruptors such as bisphenol-A (BPA). To test this hypothesis, mice were exposed to BPA and/or stigmasterol (S), an FXRα antagonist. Following the exposure to both molecules, wild-type animals showed impaired fertility and lower sperm cell production associated with the alteration of the establishment and maintenance of the undifferentiated germ cell pool. The crosstalk between BPA and FXRα is further supported by the lower impact of BPA in mice genetically ablated for Fxrα and the fact that BPA counteracted the effects of FXRα agonists. These effects might result from the downregulation of *Fxrα* expression following BPA exposure. BPA and S act additively in human testis. Our data demonstrate that FXRα activity modulates the impact of BPA on male gonads and on undifferentiated germ cell population.

INTRODUCTION

The incidence of various male reproductive disorders such as cryptorchidism, testicular cancer, and low sperm count has gradually increased in some parts of the world (Serrano et al., 2013; Skakkebaek et al., 2016). It has been hypothesized that these abnormalities might be related at least in part to increasing exposures to environmental pollutants. Among these, bisphenol-A (BPA) is one of the most thoroughly studied endocrine disruptors. It is widely used in a variety of common products including baby and water bottles, food container linings, and medical tubing (Ben Maamar et al., 2015; Carwile et al., 2009; Desdoits-Lethimonier et al., 2017; Olea et al., 1996; Vandenberg et al., 2007, 2010). Lower sperm quality and deregulation of sex hormone concentrations have been associated with BPA exposure (Rochester, 2013). In rodents, BPA exposure accelerates growth and puberty, disrupts embryonic development, and alters the process of meiosis (Liu et al., 2014; Takai et al., 2001; Vrooman et al., 2015; Xie et al., 2016). At the molecular level, BPA acts through different pathways via the binding and activation of receptors such as estrogen receptor alpha (ESR1, ERα) and estrogen receptor beta (ESRS2, ERβ) in the testis (Matthews et al., 2001; Rouiller-Fabre et al., 2015; Wetherill et al., 2007).

Recently, several studies highlight links between the estrogenic signaling pathways and the bile acid nuclear receptor farnesoid X receptor alpha (FXRα; NR1H4) (Baptissart et al., 2013). This suggests potential links between the impacts of estrogenic environmental substances and FXRα signaling pathways. Expressed in the mouse testis, FXRα regulates male fertility via several mechanisms. We thus hypothesized that interactions might exist between FXRα signaling pathways and the testicular effects of estrogenic environmental substances such as BPA. To test this hypothesis, wild-type and *Fxrα*-deficient ($Fxr\alpha^{-/-}$) mice were exposed to BPA. We also modulate the activity of FXRα using treatment with an antagonist, namely stigmasterol (S) (Carter et al., 2007). Pups were exposed *in utero* from post-coitum day 6.5 (PC6.5) and until post-partum day 5 (PP5) to BPA, S, or both substances (BS). Our data clearly support a crosstalk between FXRα signaling pathways and BPA, as highlighted by the lower effect of BPA exposure on $Fxr\alpha^{-/-}$ males. Surprisingly, in wild-type males the antagonism of FXRα using S led to additive effects with BPA resulting in altered male fertility. Moreover, we demonstrate that co-exposure to BPA and S is likely to be transposable to human testis, as BS exposure induced a decrease in Sertoli cell number and an increase in the number of undifferentiated spermatogonia. The crosstalk between BPA and FXRα was sustained by the lower impact of BPA in $Fxr\alpha^{-/-}$ males, and by the fact that BPA counteracted the effects of the FXRα agonist GW4064. These effects might in part result from the downregulation of *Fxrα* gene following BPA exposure within germ cells.

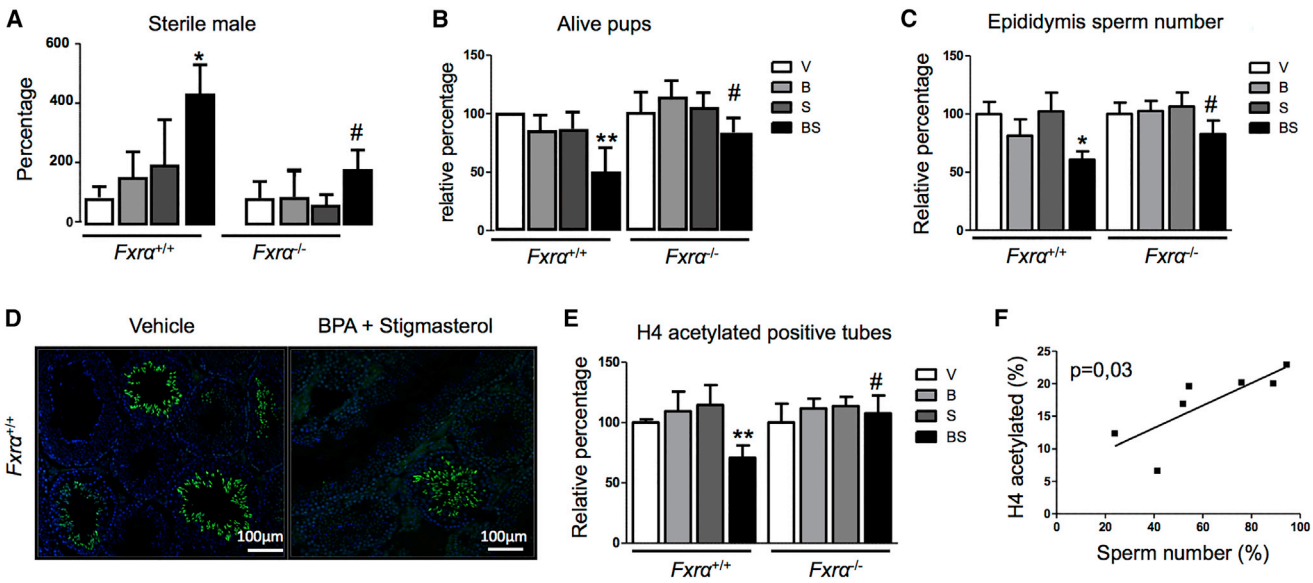

Figure 1. Co-exposure to BPA and Stigmasterol Induces Male Fertility Disorders

(A) Percentage of sterile males at 6 months (n = 7–28 per group).

(B) Relative percentage of live pups obtained (n = 7–28 per group).

(C) Relative sperm count in the epididymis head (n = 7–28 per group).

(D) H4-acetylated immunohistochemistry on 6-month-old testis $Fxr\alpha^{+/+}$ and $Fxr\alpha^{-/-}$. Scale bars, 100 µm. H4-acetylated stained spermatid cells.

(E) Relative percentage of H4-acetylated-positive tubes (n = 7–28 per group).

(F) Correlation between percentage of H4-acetylated-positive tubes and sperm number, $p < 0.05$ by two-tailed Pearson test (n = 3–17 per group).

Data are expressed as means ± SEM. In all panels: $^{*,\#}p < 0.05$ by two-way ANOVA analyses. *Difference compared with vehicle of same genotype; $^{\#}$difference between genotypes for the same treatment. $^{**}p < 0.01$. V, vehicle; B, BPA; S, stigmasterol; BS, BPA + stigmasterol.

RESULTS

Co-exposure to BPA and S Induces Male Fertility Disorders

To analyze the interactions between FXRα signaling pathways and the testicular impacts of BPA, in utero/neonatal exposures were performed on $Fxr\alpha^{+/+}$ or $Fxr\alpha^{-/-}$ mice with vehicle, BPA, the FXRα antagonist S, or BS from PC6.5 up to 5 days after birth (see Experimental Procedures). First, the impacts of treatments on male fertility were analyzed in adult male mice. No effect of the *in utero*/neonatal exposures on male fertility was noticed in 3-month-old animals (Figure S1A). In contrast at 6 months of age, 50% of $Fxr\alpha^{+/+}$ males co-exposed to BS were sterile (Figure 1A), whereas at the doses used no effect of single molecules was noticed in $Fxr\alpha^{+/+}$ mice. In addition, no effect of the exposures was observed in $Fxr\alpha^{-/-}$ males (Figure 1A). Interestingly, no difference between groups was noted regarding the reproductive capacities of males (percentage of females plugged or the percentage of plugs with fertilizations) (Figure S1B). Moreover, BS-treated $Fxr\alpha^{+/+}$ males remaining fertile generated 50% less pups compared with controls at 6 months of age (Figure 1B).

The altered fertility in $Fxr\alpha^{+/+}$ males exposed to BS was associated with a significant decrease in spermatozoa production as revealed by the sperm cell number in the head of the epididymis at 6 months of age (Figure 1C). This suggests that part of the fertility defects evidenced originates from altered spermatogenesis. No major difference in histology was seen between groups (Figure S2A). However, $Fxr\alpha^{+/+}$ males treated with BS showed fewer seminiferous tubules stained with acetylated histone H4 (H4ac), a marker of post-meiotic cells (Figures 1D and 1E). The percentage of H4ac-positive seminiferous tubules was positively correlated with the number of spermatozoa (Figure 1F). This suggests that part of the fertility defects evidenced originates from an impaired testicular process. Thus, to define the impacts of BS exposure on male fertility, testicular homeostasis of male mice was analyzed at post-natal day 10 (P10), soon after the end of the treatments, as well as in adult mice at 3 or 6 months of age.

Fetal and Neonatal Exposure to BPA and S Alters Sertoli Cell Homeostasis

Within testis, Leydig and Sertoli somatic cells are known to play major roles to ensure testicular homeostasis. At

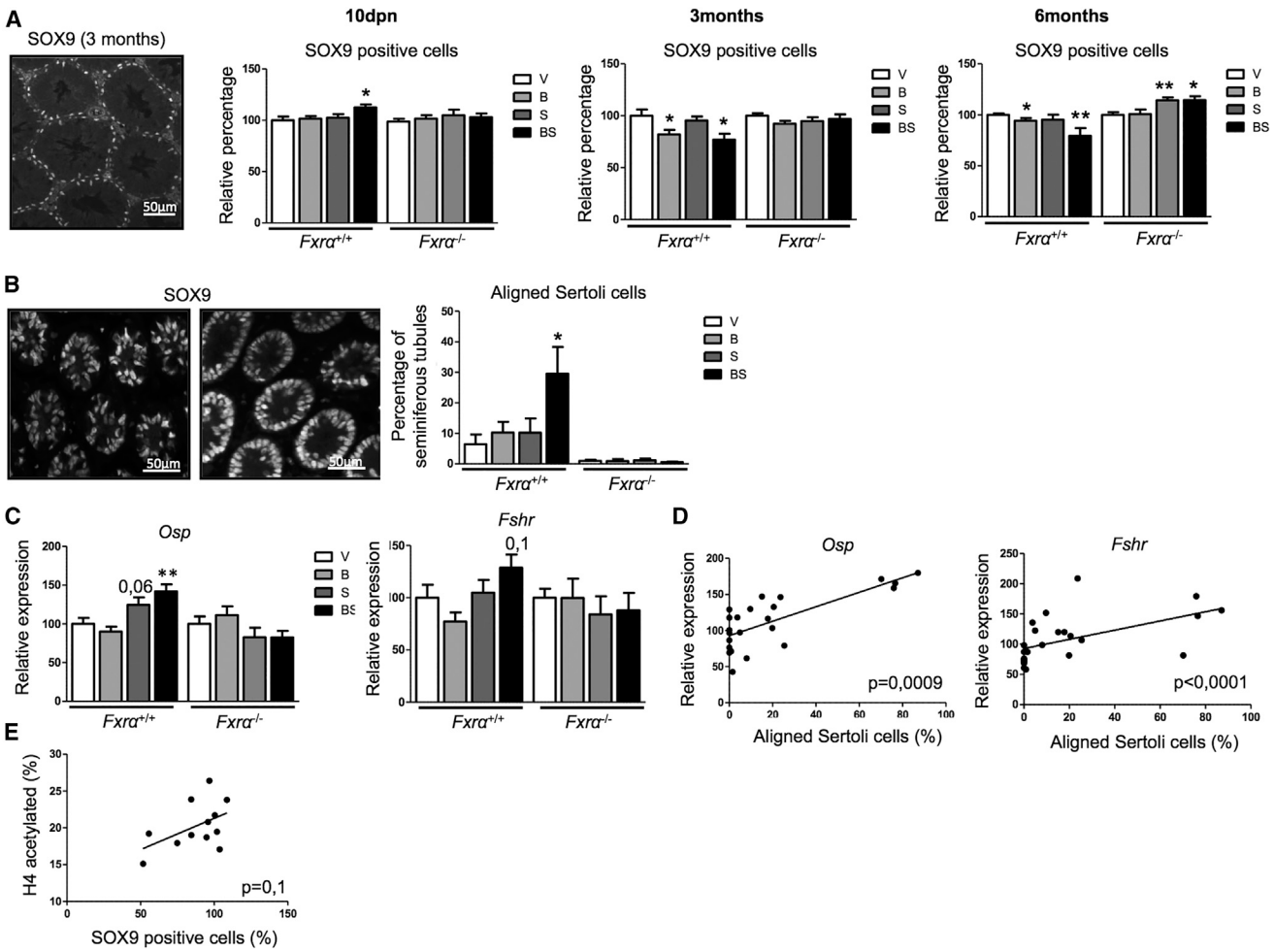

Figure 2. Co-exposure to BPA and Stigmasterol Alters Sertoli Cell Homeostasis

(A) Sox9 labeling in 3-month testis. Sox 9 is expressed in Sertoli cells. Scale bar, 100 µm. Relative percentage of sox9-positive cells per tube at post-natal day 10 (P10), 3 months, or 6 months of age (n = 6–20 per group).

(B) Sox9 immunohistochemistry on post-partum day 10 (PP10) testis. Scale bars, 50 µm. Percentage of tubes with aligned Sertoli cells.

(C) mRNA accumulation of *Osp* and *Fshr* involved in Sertoli cell function using qPCR (n = 6–14 per group).

(D) Correlation between the expression of *Osp* and *Fshr* and the percentage of tubules with aligned Sertoli cells, $p < 0.05$ by two-tailed Pearson test.

(E) Correlation between the percentage of tubes with H4-acetylated staining and the percentage of SOX9-positive cells, $p < 0.05$ by two-tailed Pearson test.

Data are expressed as means ± SEM. In all panels: *$p < 0.05$, **$p < 0.01$ by two-way ANOVA analyses. V, vehicle; B, BPA; S, stigmasterol; BS, BPA + stigmasterol.

6 months of age, no effect was observed on testosterone levels (Figure S2B), suggesting that it might not be a key factor in the altered fertility.

Interestingly, at 6 months of age, $Fxr\alpha^{+/+}$ males exposed to BPA or BS presented a significant decrease in the number of Sertoli cells, whereas BS-treated $Fxr\alpha^{-/-}$ males presented an increased number of Sertoli cells (Figure 2A). In BPA- and BS-treated wild-type males, the lower Sertoli cell number compared with vehicle-treated group was also observed in 3-month-old animals (Figure 2A). Strikingly, at P10, soon after the end of the treatment period, an increase in the number of Sertoli cells was observed (Figure 2A). In addition, at P10, BS treatment in $Fxr\alpha^{+/+}$ males led to an increased number of seminiferous tubules with Sertoli cells aligned at the basement membrane (Figure 2B). The expressions of Sertoli cell markers, such as *Osp* and *Fshr*, indicative of cell maturity, were increased in BS-treated $Fxr\alpha^{+/+}$ animals (Figure 2C) and positively correlated with the alignment of Sertoli cells (Figure 2D). These data suggest that, following BS exposure, an earlier maturation of Sertoli cells

might have led to an earlier arrest of proliferation, leading at adulthood to the lower number of Sertoli cells in the BS-treated group compared with the control group. These data were supported by a trend of a lower Sertoli cell proliferation rate at P10 in BS-treated males compared with the control group (Figure S2C). BS treatment clearly altered Sertoli cell homeostasis from neonates to adulthood; however, no correlation was noticed between Sox9-positive cell number and the number of H4ac-positive seminiferous tubules (Figure 2E). This highlights that, even if they might be involved, the altered number of Sertoli cells was not a key factor for the deleterious effects of BS exposure on fertility.

Fetal and Neonatal Exposure to BPA and S Alters Adult Germ Cells Physiology

As fertility disorders were associated with altered spermatogenesis (correlation between the number of H4ac-positive seminiferous tubules and sperm cell number, Figure 1F), we thus decided to explore spermatogenesis. In 6-month-old *Fxrα*$^{+/+}$ males exposed to either S or BS, testis showed fewer seminiferous tubules stained for PLZF, a specific marker of undifferentiated spermatogonia (Figure 3A). In PLZF-positive seminiferous tubules, fewer PLZF-positive cells were observed in *Fxrα*$^{+/+}$ males exposed to BS (Figure 3B). The combination of these indicators showed that, at 6 months of age, there was a decrease in the number of undifferentiated spermatogonia per total seminiferous tubules in *Fxrα*$^{+/+}$ males exposed to BS (Figure 3C). In *Fxrα*$^{-/-}$ mice, no difference between groups was noted (Figures 3A–3C). Interestingly, it appears that the altered sperm cell production originated in the early step of spermatogenesis, as highlighted by the correlation between the number of PLZF-positive seminiferous tubules and the number of spermatozoa (Figure 3D). This result was supported by the fact that a similar correlation was observed between the numbers of PLZF-positive seminiferous tubules and the percentage of H4ac-positive seminiferous tubules (Figure 3E). This suggests that fertility disorders following exposure to BS found its origin in early step of spermatogenesis. Interestingly, at 3 months of age, mice exposed to BS showed an increased number of PLZF-positive undifferentiated spermatogonia (Figures 3A–3C). Surprisingly, even with more PLZF-positive cells, at this age a lower mRNA accumulation of *Plzf* and *Nanog* was observed in BS-exposed Fxrα$^{+/+}$ males (Figure 3F).

Fetal and Neonatal Exposure to BPA and S Alters Neonatal Germ Cells Physiology

In line with the *in utero*/neonatal exposure, we went back to the early impacts of the treatments on the PLZF-positive population. Strikingly, in P10 male mice, there was a dual effect of BS treatment. Indeed, a lower number of PLZF-positive seminiferous tubules was noticed (Figure 4A); whereas an increased number of PLZF-positive cells per-positive tubule was observed in BS-treated Fxrα$^{+/+}$ males compared with the control group (Figure 4B).

As histological variations were observed between groups (alignment of Sertoli cells [Figure 2B] and number of undifferentiated spermatogonia [Figure 4A]), we performed correlation analyses on P10 testis, between gene expressions and phenotypes to determine the important pathways (Figure 4C). As expected, a positive correlation was observed between *Plzf* mRNA accumulation and the number of PLZF-positive seminiferous tubules (Figure 4C). In addition, a positive correlation was also observed for genes involved in meiosis such as *Stra8*, *Dmc1*, and *Sycp3* and number of undifferentiated spermatogonia (Figure 4C). Interestingly, the number of PLZF-positive cells was also correlated with the alignment of Sertoli cells (Figure 4D). Consistently, a negative correlation was observed for genes involved in meiosis, such as *Stra8*, *Dmc1*, and *Sycp3*, and the percentage of seminiferous tubules with aligned Sertoli cells (Figure 4E). This suggests that paracrine factors might come into play in response to BS, resulting in functional interactions between Sertoli cells and undifferentiated germ cells leading to altered differentiation through meiosis.

We then defined that the mRNA accumulation of *Cyp26b1*, a major regulator of meiosis via the inhibition of the retinoid pathway, was negatively correlated with the number of undifferentiated spermatogonia (Figure 4F) and positively correlated with the percentage of seminiferous tubules with aligned Sertoli cells (Figure 4G). This was in agreement with the expression of *Shp*, a known repressor of meiosis, which was negatively correlated with the number of undifferentiated spermatogonia (Figure 4F) and positively correlated with the percentage of seminiferous tubules with aligned Sertoli cells (Figure 4G). All these data are consistent with an alteration of meiosis after BS treatment. The alterations of the initial steps of spermatogenesis with the PLZF-positive population in BS-treated animals might participate in the altered testicular histology at adulthood.

Interestingly, we have identified that *Dnmt3l* mRNA accumulation, a known regulator of PLZF, was increased in response to BS in *Fxrα*$^{+/+}$ male mice (Figure S3A). From the mechanistic point of view, it has been reported that DNMT3L is important for the stability of PLZF (Liao et al., 2014). Thus this overexpression of *Dnmt3l* in BS-treated Fxrα$^{+/+}$ males, by stabilizing PLZF in PLZF-positive seminiferous tubules, could participate to the altered spermatogenesis, as ectopic overexpression of PLZF has been shown to inhibit germ cell differentiation (Ferder and Wang, 2015).

Co-exposure to BPA and S Induces Human Testis Histology Defects

To assess whether some of the data obtained on mouse testis could be transposable to human testis, an *ex vivo* model system was used. Adult testis explants were first

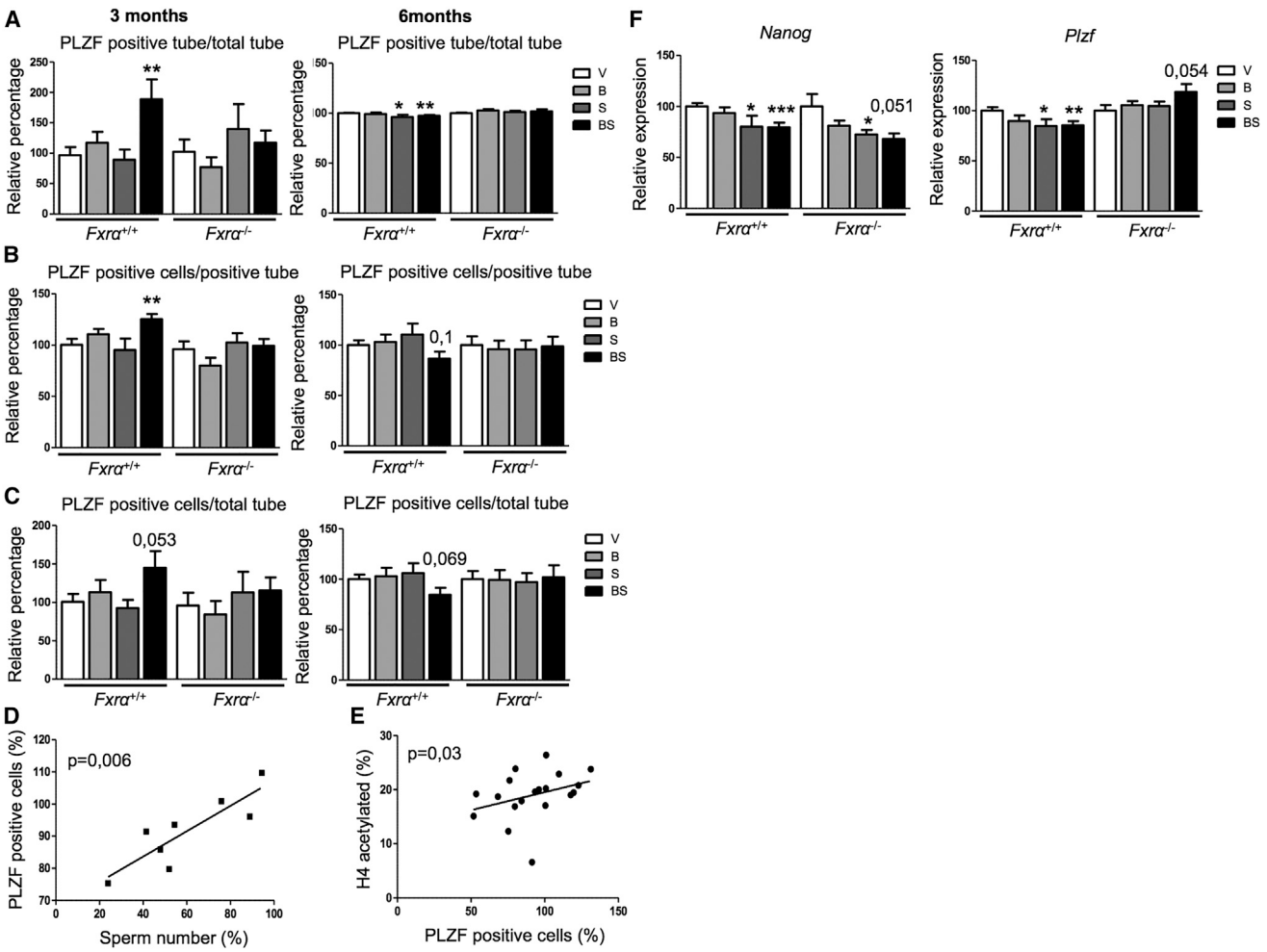

Figure 3. Co-exposure to BPA and Stigmasterol Alters Germ Cell Physiology

(A) Relative percentage of PLZF-positive tubes relative to total tube at 3 and 6 months.

(B) Relative percentage of PLZF-positive cells relative to positive tube at 3 and 6 months.

(C) Relative percentage of PLZF-positive cells relative to total tube at 3 and 6 months.

(D) Correlation between the percentage of PLZF-positive cells and sperm number, $p < 0.05$ by two-tailed Pearson test.

(E) Correlation between the percentage of PLZF-positive cells and percentage of tubes with H4-acetylated staining, $p < 0.05$ by two-tailed Pearson test.

(F) mRNA accumulation of *Nanog* and *Plzf*, using qPCR (n = 6–14 per group).

Data are expressed as means ± SEM. In all panels: *p < 0.05, **p < 0.01, ***p < 0.001 by two-way ANOVA analyses. V, vehicle; B, BPA; S, stigmasterol; BS, BPA + stigmasterol.

exposed for 48 hr with several doses of either BPA or S (Figures S3B and S3C). No effect was observed for the 10^{-5} M dose. The exposure to S did not affect the number of Sertoli cells. Regarding the analysis of undifferentiated spermatogonia (PLZF-positive cells), no effect was noted following exposure to either BPA or S alone (Figure S3C).

We then decided to study the effects of the BPA and S combination using BPA at 10^{-8} M concomitantly with S at 10^{-5}M. Interestingly, a significant decrease in Sertoli cell number was observed (Figures 5A and 5B). In line with the impact of BS exposure on Sertoli cell number, the concentration of INHIBIN-B was increased following BS exposure (Figure 5C). BPA or S alone did not show any impact on INHIBIN-B concentration (Figure S3D), showing the additive effects of both molecules on testis function. In addition, a significant BS-induced increase in the number of PLZF-positive undifferentiated spermatogonia was noted (Figures 5D and 5E). These data demonstrate that, as in the mouse, BPA and S interfere with the integrity of the human testis structure and functions.

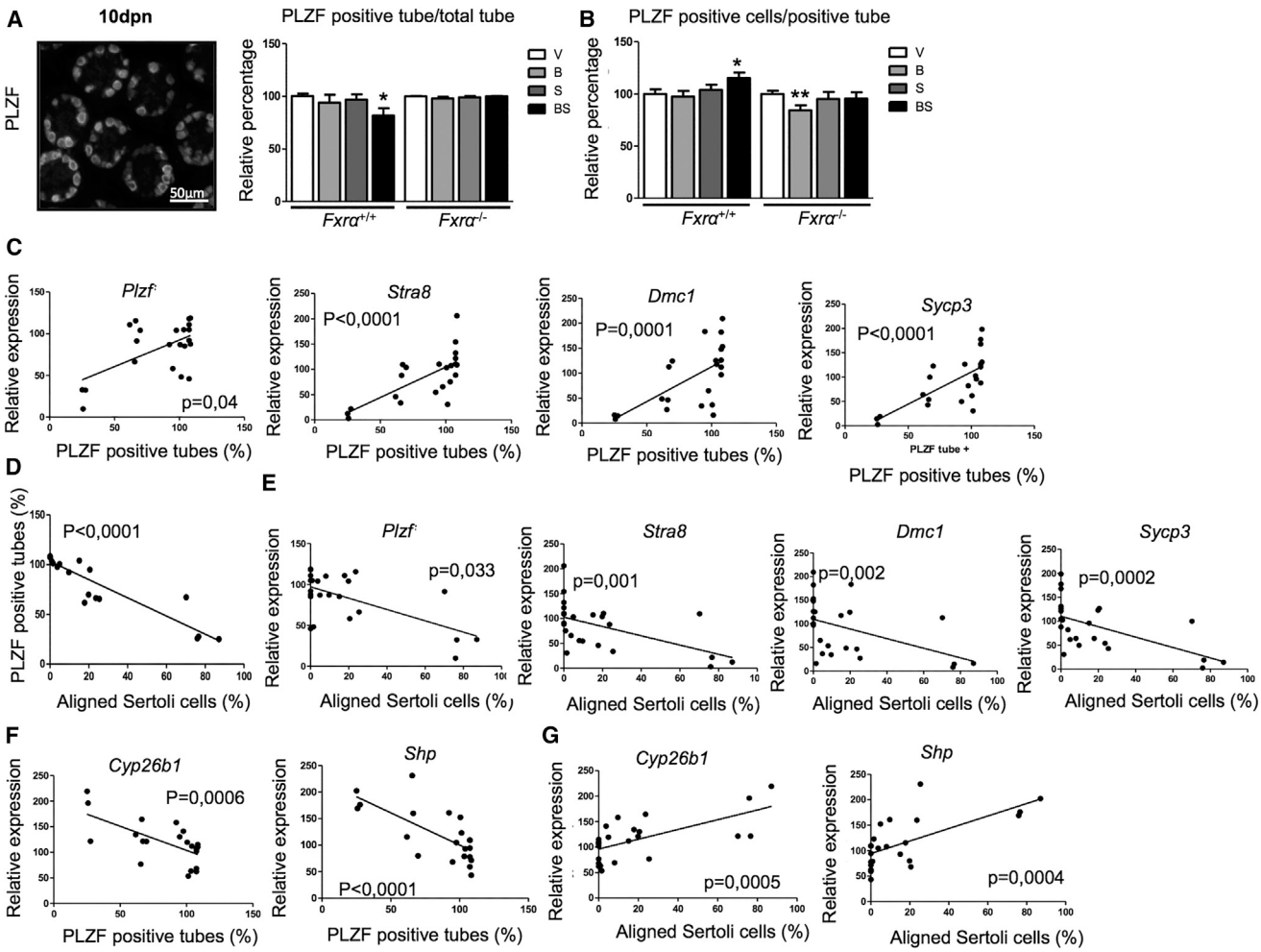

Figure 4. Fetal and Neonatal Exposure to BPA and Stigmasterol Induces Germ Cell and Defects at PP10

(A) PLZF immunohistochemistry on PP10 testis. PLZF is expressed in undifferentiated spermatogonia. Scale bar, 50 µm. Relative percentage of PLZF-positive tubes relative to total tubes.

(B) Relative percentage of PLZF-positive cells per tube.

(C) Correlation between the expression of germ cell markers *Plzf*, *Stra8*, *Dmc1*, and *Scp3*, and the percentage of PLZF-positive tubules, $p < 0.05$ by two-tailed Pearson test.

(D) Correlation between the percentage of PLZF-positive tubes and the percentage of tubules with aligned Sertoli cells, $p < 0.05$ by two-tailed Pearson test.

(E) Correlation between the expression of *Cyp26b1*, *Shp*, and the percentage of tubules with aligned Sertoli cells, $p < 0.05$ by two-tailed Pearson test.

(F and G) Correlation between the expression of *Cyp26b1* and *Shp* and the percentage of PLZF-positive tubules (F). Correlation between the expression of *Cyp26b1* and *Shp* and the percentage of the percentage of tubules with aligned Sertoli cells (G).

Data are expressed as means ± SEM. In all panels: *$p < 0.05$, **$p < 0.01$ by two-way ANOVA analyses. For correlation: $p < 0.05$ by two-tailed Pearson test (n = 6–14 per group). V, vehicle; B, BPA; S, stigmasterol; BS, BPA + stigmasterol.

As in mouse, testosterone may not be involved in the impact of co-exposure to BS on the altered number of Sertoli and undifferentiated germ cells. Indeed, co-exposure did not impact testosterone levels in the media of human testis explants (Figure 5F). Consistently, the lack of impact of co-exposure to BS on Leydig cells was confirmed with the measurement of INSL3 (Figure 5G).

Modulation of FXRα Activity Impacts the Testicular Sensitivity to BPA

We then studied the molecular mechanisms in order to explain how BPA and S transduced their cooperative effects in an FXRα-dependent manner at P10. As BPA has estrogenic activity, we first analyzed the expression of several receptors known to mediate the effects of BPA. No difference

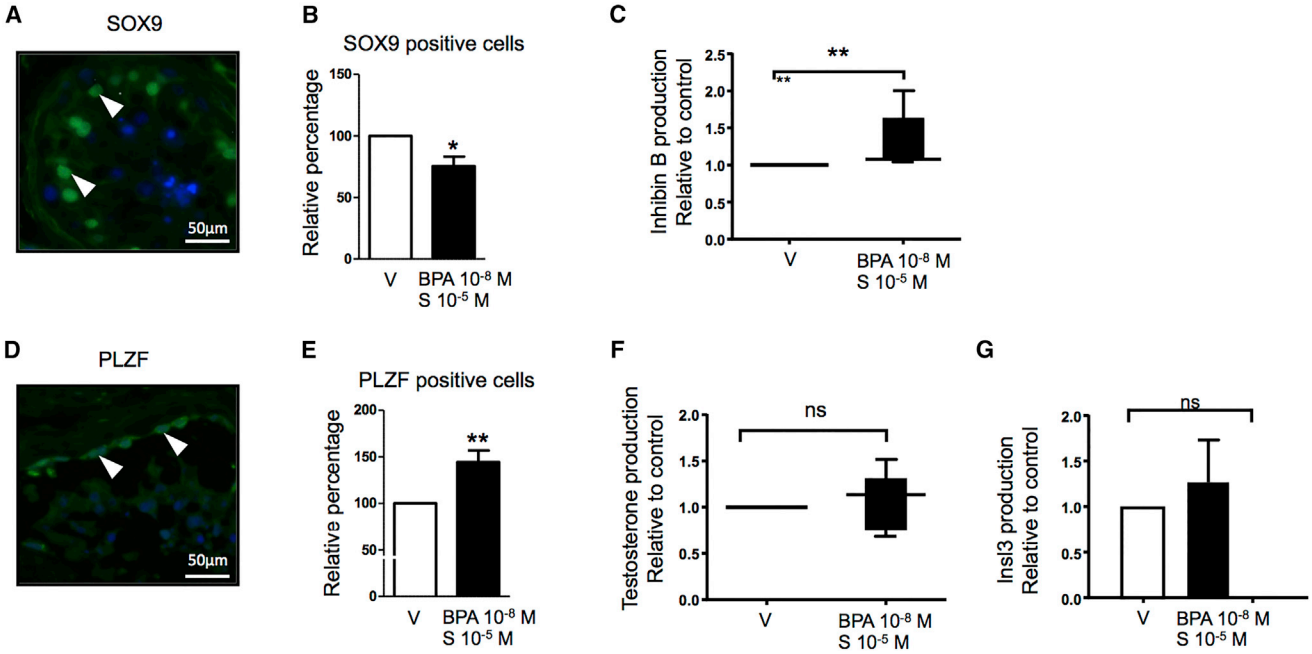

Figure 5. Co-exposure for 48 hr to BPA and Stigmasterol Induces Defects in Human Adult Testis

(A) SOX9 labeling in human adult testis. Arrowheads show stained cells. Scale bar, 50 µm.

(B) Relative percentage of SOX9-positive cells per mm^2.

(C) INHIBIN-B levels in media of cultured explants were co-exposed for 48 hr to stigmasterol (10^{-5} M) and BPA (10^{-8} M).

(D) PLZF labeling in human adult testis. Arrowheads show PLZF (B) stained cells. Scale bar, 50 µm.

(E) Relative percentage of PLZF-positive cells per mm^2 (n = 5–6 per group).

(F) Testosterone levels in media of cultured explants co-exposed for 48 hr to stigmasterol (10^{-5} M) and BPA (10^{-8} M).

(G) INSL3 levels in media of cultured explants co-exposed for 48 hr to stigmasterol (10^{-5} M) and BPA (10^{-8} M).

*p < 0.05 by two-tailed unpaired Student's t test. V, vehicle; S, stigmasterol. Values are means ± SEM of 3–11 independent experiments from different donors. Dose responses were analyzed for significance with the Mann-Whitney test. **p < 0.01. ns, not significant. Slopes (β) and p values of Spearman correlations are indicated. C, control.

in *Esr2* (*Erβ*) expression was noted upon treatments (Figure S4A). However, as previously reported (Martinot et al., 2017a), a lower mRNA accumulation of *Esr2* was observed in *Fxrα*$^{-/-}$ compared with *Fxrα*$^{+/+}$ males (Figure S4A). This might participate to the lower effects of BPA in Fxrα$^{-/-}$ males as for meiotic gene expression (Figure S4B). In addition, an increase in *Esr1* (*Erα*) mRNA accumulation was noted in BS-exposed *Fxrα*$^{+/+}$ males compared with controls (Figure S4A). Interestingly, BPA exposure alone increased *Esr1* mRNA accumulation in *Fxrα*$^{-/-}$ (Figure S4A). To validate the functionality of such mRNA dysregulation of *Esr1*, we have then explored the expression of ESR1 target genes, among which were *Insl3*, *Cyp19*, *Star*, *Cyp17a1*, and *Greb1* (Volle et al., 2009). However, no effect was observed following BS exposure, suggesting that the alteration of Esr1 was not transduced to an altered signaling pathway (Figure S4C).

To ensure the involvement of FXRα in the effects of BS exposure, we analyzed the expression of some of its target genes such as *Bsep*, *Dax-1*, *G6pase*, *Pepck*, and *ApoA1* in *Fxrα*$^{+/+}$ and *Fxrα*$^{-/-}$ males (Kemper, 2011; Yang et al., 2017). We analyzed the impacts of BPA, ST, and BS on these genes. Interestingly, mRNA accumulations of all these genes were altered (Figure S5). Results clearly demonstrated the involvement of FXRα as no effect was observed in Fxrα$^{-/-}$ males (Figure S5A). These data supported the occurrence of crosstalk between BPA and FXRα signaling pathways. Indeed, effects were observed only in the context of combined exposures, and not with exposure to single molecules (Figure S5A). However, the chronic exposure might be associated with complex mechanisms as some negative FXRα target genes, namely *Apoa1*, *G6pase*, and *Pepck*, were upregulated, whereas target genes such as *Bsep* and *Dax-1* were increased too (Figure S5A). The use of RNA sequencing (RNA-seq) (see Data S1) clearly demonstrates that the effects of S were mainly lost in *Fxrα*$^{-/-}$ males (Figure S5B). More surprisingly, most gene regulations by BPA were also lost in *Fxrα*$^{-/-}$ males compared with *Fxrα*$^{+/+}$ males. This clearly highlights the main role of FXRα in some of the testicular impacts of BPA (Figure S5B).

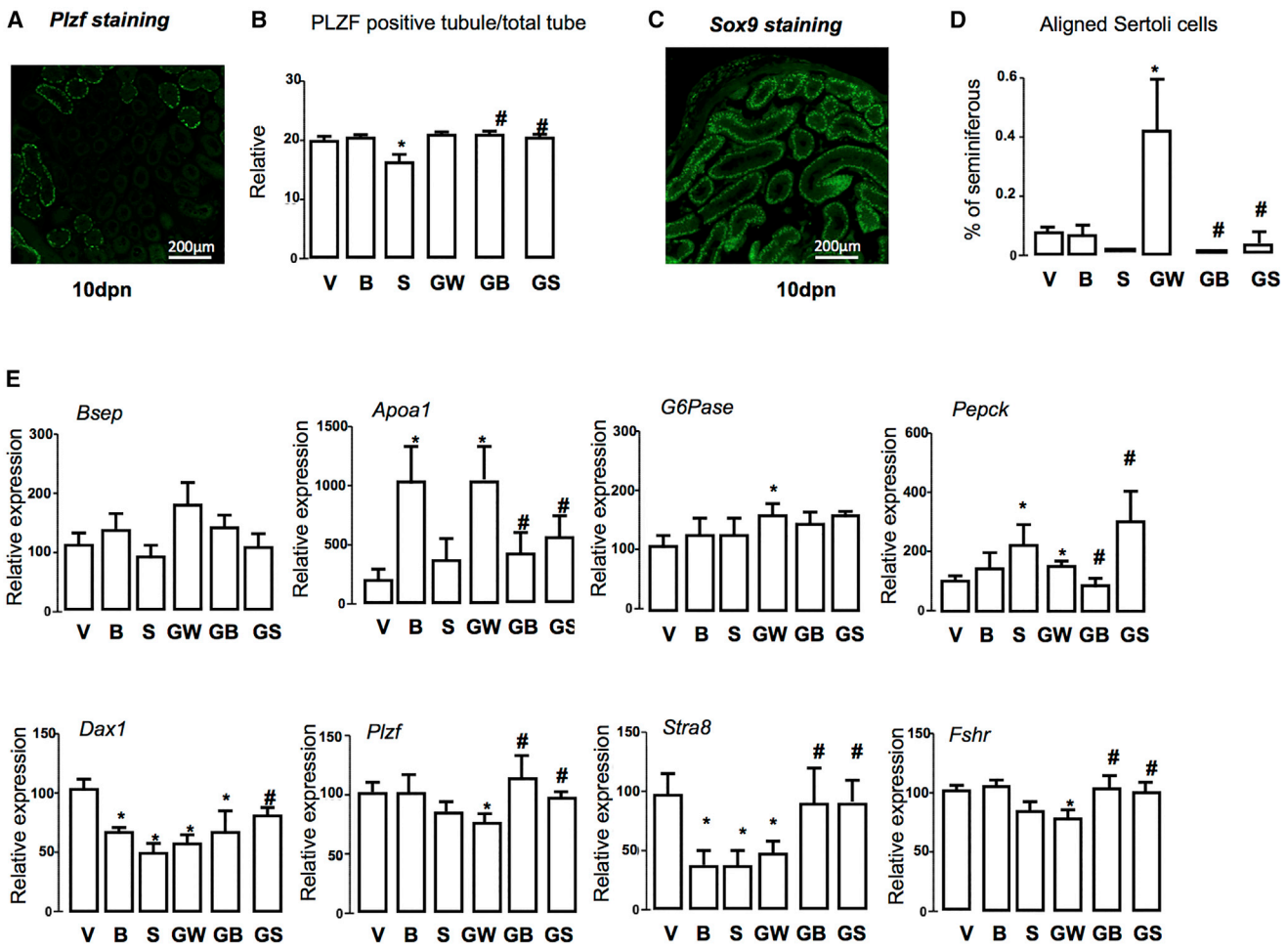

Figure 6. FXRα Signaling Pathway Induces Germ Cell and Defects at PP10

(A) PLZF immunohistochemistry on PP10 testis is expressed in undifferentiated spermatogonia. Scale bar, 50 µm.

(B) Relative percentage of PLZF-positive tubes relative to total tubes in mice treated with vehicle, B, S, GW4064, GWB, or GWS.

(C) Sox9 immunohistochemistry on PP10 testis. Scale bar, 50 µm.

(D) Percentage of tubes with aligned Sertoli cells in mice treated with vehicle, B, S, GW4064, GB, or GS.

(E) mRNA accumulation of *Bsep*, *Apoa1*, *G6Pase*, *Pepck*, *Dax-1*, *Plzf*, *Stra8*, and *Fshr* in testis of mice treated with vehicle, B, S, GW4064, GWB, or GWS using qPCR (n = 8–14 per group).

Data are expressed as means ± SEM. In all panels: $^{*,\#}p < 0.05$ by two-way ANOVA analyses. *Difference compared with vehicle; #difference compared with GW-treated group. V, vehicle; B, BPA; S, stigmasterol; BS, BPA + stigmasterol; GW, GW4064; GB, GW4064 + BPA; GS, GW4064 + S.

To add evidence that FXRα was critical in the observed effects, we then performed additional experiments using GW4064, an FXRα agonist, combined with S or BPA. Indeed, one might think that, if BPA and the FXRα antagonist share common signaling pathways, both S and BPA might interact with the signaling of the FXRα agonist (GW4064). Male mice were thus exposed to vehicle, GW4064, BPA, GW4064 + BPA (GB), or GW4064 + S (GS). Histological analyses of P10 testis demonstrate that the animals of the GW4064-treated group showed a lower number of PLZF-positive seminiferous tubules (Figures 6A and 6B). As expected, the effects of the FXRα agonist GW4064 were not observed in *Fxrα*$^{-/-}$ males (Figure S6A). Interestingly, in *Fxrα*$^{+/+}$ animals, co-treatment with S counteracted the effects of GW4064 (Figure 6B). All these data clearly demonstrate the main role of FXRα in the observed phenotypes.

Consistently with the crosstalk between BPA and FXRα signaling as suggested by the effects of BS exposure, the histological analyses demonstrate that BPA exposure abolished the impacts of GW4064 on the number of PLZF-positive seminiferous tubules (Figure 6B). These data clearly demonstrate the main role of FXRα in the observed phenotypes.

Our data showed that BS exposure also altered Sertoli cell homeostasis with an earlier alignment to the basal

membrane. The use of GW4064 led to similar effects (Figures 6C and 6D). Interestingly, as for PLZF cells, GW4064 exposure mimicked the observed effects of BS treatment, with an increase of the percentage of seminiferous tubules with aligned Sertoli cells at the basal membrane compared with the control group (Figure 6D). As expected, the effects of the FXRα agonist GW4064 were not observed in *Fxrα*$^{-/-}$ males (Figure S6B) Moreover, BPA and S were able to counteract the impact of GW4064 on Sertoli cells.

We then moved to molecular analyses to see whether GW4064 can have similar targets to BS. We thus analyzed the mRNA accumulation of genes such as *Bsep*, *Dax-1*, *G6pase*, *Pepck*, and *ApoA1* as described previously. Data showed that, in response to GW4064 exposure, and as observed in BS-treated animals, the mRNA accumulation of most of these genes were upregulated, excepted for Dax-1, which was downregulated in response to GW (Figure 6E). As expected, the effects of the FXRα agonist GW4064 were not observed in *Fxrα*$^{-/-}$ males (Figure S6C). The crosstalk between the BPA and FXRα signaling pathways was highlighted by the fact that either B or S counteracted the effects of the FXRα agonist for these genes, such as *Apoa1*, *Pepck*, and *Dax-1* (Figure 6E).

To confirm the observations suggesting that BS and GW4064 exposures could have similar chronic effects, we then analyzed other genes affected by BS in P10 testis. As previously seen with BS, GW404 altered the expression of *Fshr*, *Plzf*, and *Stra8* (Figure 6E). In addition, both B and S counteracted the effects of the FXRα agonist (Figure 6E). As expected, the effects of the FXRα agonist GW4064 were not observed in *Fxrα*$^{-/-}$ males (Figure S6C).

Overall, it was quite unexpected that similar histological and molecular impacts could be observed between GW4064-treated animals and animals exposed to BS. This might be relevant in order to explain the involved molecular mechanisms.

It has been previously demonstrated that FXRα controls the germ cell fate during post-natal development; this is consistent with the observed FXR-dependent impacts of BS and GW4064 exposure. Indeed, the abnormal expression of genes known to be involved in maintenance of undifferentiated germ cells, such as *Plzf* (Figure 4C), might be part of the initiating step of the altered spermatogenesis observed later at 6 months of age. To better understand the involved mechanisms, we decided to study the cellular expression of *Fxrα* with post-natal testis. Data showed that Fxrα is expressed in undifferentiated spermatogonial cells as demonstrated using THY-1 magnetic cell sorting (Figure 7A). We thus decided to decipher the impact of BS exposure specifically using the spermatogonial cell line GC1-spg. Interestingly, using cell culture experiments with the spermatogonial cell line GC1-spg, we have demonstrated that exposure to BS led to a decrease of *Lin28*, *Nanog*, and *Gfra1* mRNA accumulations (Figure 7B).

Interestingly, from a molecular point of view, data showed that, in GC1-spg, BPA or S exposure led to lower mRNA accumulation of Fxrα mRNA, and this effect was more pronounced in co-treated cells (Figure 7C). The effect of BPA seemed to be mediated by ESR1 as suggested by the use of ICI, an Esr1 antagonist (Figure S6D). This repression of *Fxrα* by BPA and S must lead to lack of FXRα on the 5′ regulatory sequences of its target genes. This must be associated with the lack of co-repressors on the promoters and then overexpression of genes. It is indeed interesting to note that, as observed *in vivo*, *in vitro* experiments sustain data showing that FXRα activation or FXRα inhibition could lead to similar molecular events. This is highlighted here by the mRNA accumulation of *Shp* in GC1-spg cells, which was increased in response to GW4064 or in condition of small interfering RNA (siRNA) compared with vehicle-treated cells or si-Ctrl groups (Figures 7D–7F). In the same lane, consistent observations were made for *Nanog* and *Gfra1*. The mRNA expressions of *Nanog* and *Gfra1* were decreased by GW4064 as well as in si-Fxrα-transfected cells compared with si-Ctrl conditions (Figures 7D–7F). Moreover, this impact of the GW4064 exposure was reversed by either BPA or S exposure (Figures 7D–7F). Interestingly, *Gfra1* was also altered by BS exposure in an FXRα-dependent manner (Figure S6E).

All these data led us to hypothesize that, within spermatogonia, BPA might act in part through the downregulation of *Fxrα* expression, and that S might mainly act as direct antagonist of FXRα. Such an hypothesis must explain the synergistic impact of BS exposure through the lower efficiency of FXRα signaling pathways due to its lower expression (BPA) and its activity (S). To validate this hypothesis we performed additional experiments. If S is an antagonist it must inhibit the effect of GW4064 when administered concomitantly, but not necessarily if cells were pretreated for 12 hr with S. Our data confirmed this on expression of the canonical FXRα target gene *Shp*, as S abolished the effect of the GW4064 when it was administered concomitantly for 12 hr (Figures 7E and 7F).

In contrast, if BPA acts through the inhibition of *Fxrα* expression, it must be efficient only when cells will be pre-treated with BPA. Data confirmed this hypothesis as the effect of GW4064 exposure was reversed only in cells pre-treated for 12 hr with BPA before GW4064 exposure (Figures 7E and 7F).

Results obtained for *Nanog* and *Gfra1* were slightly different even if they validate the crosstalk between BPA and FXRα signaling pathways. Regarding *Gfra1*, its expression was decreased by GW4064, and S reversed this effect in both treatment conditions (co- or pre-treated cells). As

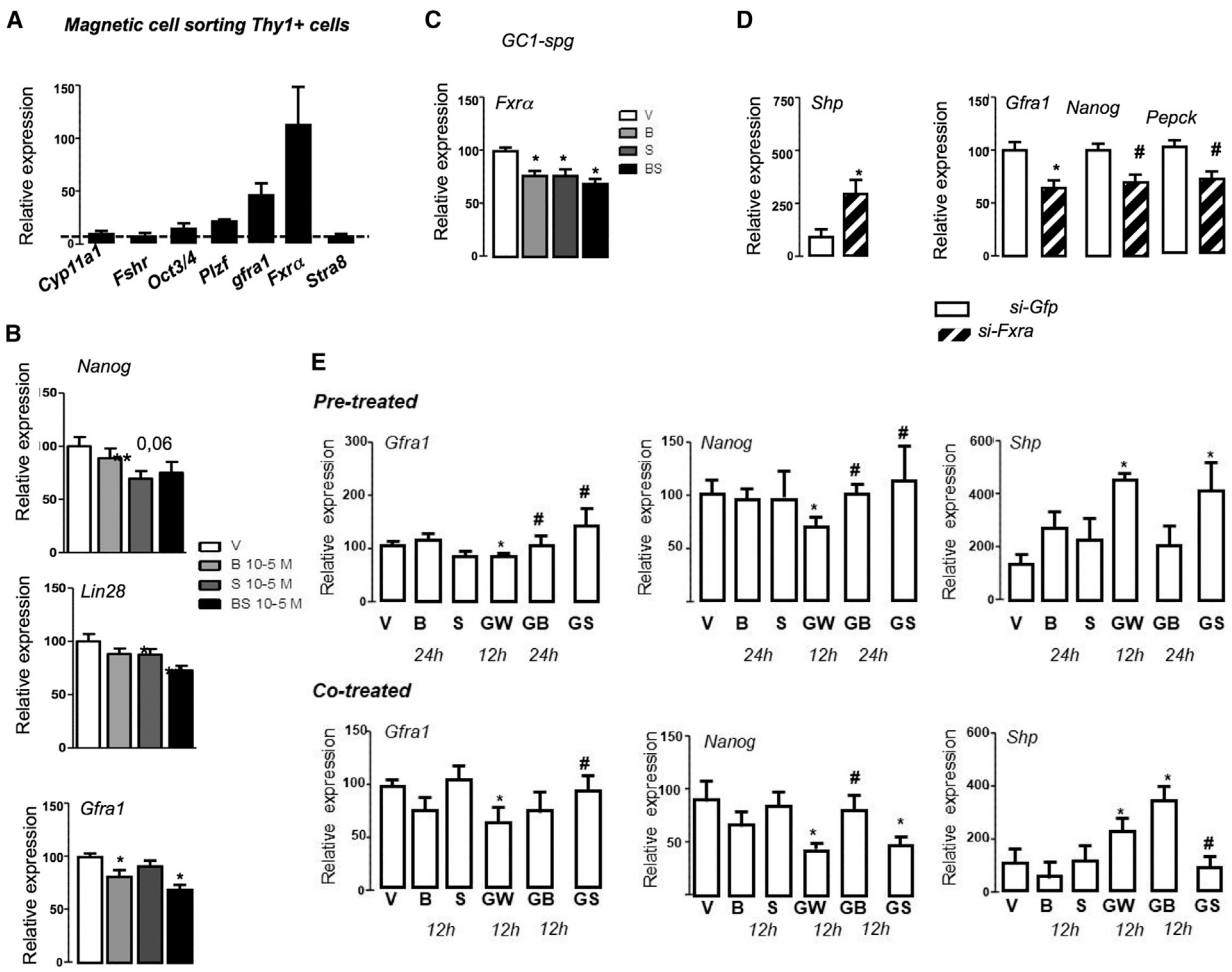

Figure 7. FXRα Signaling Pathway Acts within Germ Cells

(A) mRNA accumulation of *Cyp11a1*, *Fshr*, *Oct3/4*, *Plzf*, *Gfra1-1*, *Fxra*, and *Stra8* in undifferentiated Thy1+ cells obtained from magnetic cell sorting from P10 testis.

(B) mRNA accumulation of *Nanog* and *Gfra1* in GC1-spg spermatogonial cell line treated with vehicle, B, S, or BS (n = 6–12 per group).

(C) mRNA accumulation of *Fxr* in GC1-spg spermatogonial cell line treated with vehicle, B, S, or BS (n = 6–12 per group).

(D) mRNA accumulation of *Shp*, *Nanog*, and *Gfra1* in GC1-spg cells transfected with a si-Gfp or a siRNA directed against Fxrα (si-Fxrα) (n = 6–12 per group).

(E) mRNA accumulation of *Gfra1*, *Nanog*, and *Shp* in GC1-spg spermatogonial cell line treated with vehicle, B, S, GW, GB, or GS. For GS and GB, cells were either concomitantly treated with G and S or G and B for 12 hr, or pre-treated for 12 hr with B or S before treatment with GW for 12 hr (n = 6–12 per group). These data represent at least triplicate experiments.

Data are expressed as means ± SEM. In all panels: $^{*,\#}p < 0.05$, $^{**}p < 0.01$ by two-way ANOVA analyses. *Difference compared with vehicle; #difference compared with GW-treated group. V, vehicle; B, BPA; S, stigmasterol; BS, BPA + stigmasterol; GW, GW4064; GB, GW4064 + BPA; GS, GW4064 + S.

expected, BPA counteracted the effect of GW4064 only when pre-administered (Figures 7E and 7F).

Data obtained for *Nanog* regulation were more surprising (Figures 7E and 7F). Indeed, S was not able to inhibit the effect of GW4064 when concomitantly administered for 12 hr; whereas the inhibition was observed after pre-treatment exposure (Figures 7E and 7F). These results on *Nanog* were quite intriguing and unexpected and might reveal multiple ways of action of S to counteract FXRα activity. This could be sustained by the observation that S also regulates the expression of Fxrα gene. It could also be hypothesized that genes were regulated in sequential manner depending on specific dynamic of FXRα. As for S, it was surprising to observe that *Nanog* expression occurs differently than *Shp* and *Gfra1* following BPA and GW4064 exposure. Indeed, the impact of GW4064 on *Nanog* mRNA

accumulation was reversed by BPA as soon as co-exposure. These data also suggest other potential mechanisms explaining the crosstalk between FXRα and BPA. It might be expected that FXRα regulates the expression of intermediate(s) factor(s) that is(are) negative regulator(s) of *Nanog* and/or *Gfra1*.

DISCUSSION

We demonstrate here that, in mice, co-exposure to BPA, a well-known substance with endocrine disruptive properties, and S, a natural FXRα antagonist, induced enhanced abnormalities in male reproductive function compared with the single administration of either BPA or S. This co-exposure led to a decreased fertility in adult male mice as a result of altered testicular integrity associated with defects of spermatogenesis and lower sperm production compared with controls. Most interestingly, these indicators were almost unaffected in male mice deficient for the *Fxrα* gene. This suggests that FXRα plays an important role in testicular pathophysiology induced by co-exposure to BPA and S, and that there is a link between BPA and the FXRα signaling pathways. Consistent with this, we were also able to evidence adverse effects of this co-exposure to BPA + S on the human testis in *ex vivo* experiments. Note that the ages at which the testes were exposed to the chemicals of interest were different in the mouse and the human samples.

Communications between cell types are essential for establishing and maintaining testis structure and functions (Smith et al., 2015). It is now well established that paracrine factors between Sertoli cells and undifferentiated spermatogonia are involved in the establishment and spermatogonial stem cell self-renewal. This highlights the critical need to work with integrative approaches to define the adverse effects of substances. Here, as far as the endpoint (fertility) is concerned, it appears that the alteration of Sertoli cells might participate, at least on the early impact of BS treatment on the establishment of the undifferentiated spermatogonia population, but this was not sufficient, as is highlighted by the lack of correlation between SOX-9-positive cells and the number of H4ac-positive seminiferous tubules at adulthood.

In contrast, the present data show that the chemically induced adult defects in spermatogenesis were associated with an altered number of undifferentiated spermatogonia (PLZF positive), a lower number of spermatids (H4ac positive), and a lower sperm production. As *Plzf* is expressed in undifferentiated spermatogonia in the testis, and is required to regulate self-renewal and maintenance of the stem cell pool, PLZF alteration might contribute to the altered spermatogenesis in adult mice. This primordial role is supported by the phenotype of *Plzf*-deficient male mice, which present progressive germ cell loss and testis atrophy causing infertility (Buaas et al., 2004; Costoya et al., 2004).

Interestingly, at the cellular and molecular levels, some of the deleterious effects were either BPA or S induced, and the combination of concomitant effects might explain the appearance of fertility disorders (Figure S7).

At the molecular level, our data suggest potential mechanisms of the crosstalk between FXRα and BPA. It is interesting to note that BPA had a lesser effect in the Fxrα−/− males. This might be in part due to the lower mRNA accumulation of *Esr2* observed in the testis of Fxrα-deficient mice (Figure S4B) (Martinot et al., 2017a). In addition, our data also demonstrate that BPA expression decreased the expression of Fxrα with germ cell lineage. This is consistent with recent data demonstrating that *in utero* exposure to BPA decreased *Fxrα* expression in the liver (Susiarjo et al., 2017). Overall, these data show that FXRα is an unexpected mediator of BPA effects.

In that line, the synergistic impacts of BS exposure might rely, at least in part, on the decreased efficiency of FXRα signaling pathways due to its lower expression (BPA) and its activity (S). Indeed, phenotypic and molecular signatures are similar in BS- or GW4064-exposed mice. In that line, it was previously demonstrated that, in the absence of ligands, nuclear receptors are fixed on the 5′ regulatory sequences on the target genes; the agonist exposure leads in turn to recruitment of co-activators and gene expression. On the other hand, gene deficiency encoding for nuclear receptor is associated with a lack of the nuclear receptor and of the co-repressors on the 5′ regulatory sequences on the target genes, and could in in turn lead to de-repression of the gene expression. These molecular properties lead to similar gene regulation in particular genes as observed here for *Shp*. This conclusion was validated using cell culture experiments on the spermatogonial cell line GC1-spg using agonist- and siRNA-based experiments. It is interesting to note that BPA and S were able to counteract the effects of GW4064. This clearly confirmed the links between FXR and BPA signaling pathways.

The kinetic of the data observed in cell culture experiments on *Nanog* expression and the crosstalks between BPA and GW4064 in one hand, and on the other hand between GW4064 and S suggest that other mechanisms might exist and will need to be further defined. However, as far as defined so far BPA does not modulate directly FXRα activity as ligand (Sui et al., 2012).

It could be suggested that FXRα regulates the expression of negative regulator(s) of *Nanog* and *Gfra1* in a similar way to *Shp* under conditions of GW6064 and BS treatment, as well as in Fxrα$^{-/-}$ mice.

A remaining question will be to define the endogenous/physiological ligand(s) of FXRα within the testis. Evidence was previously given that, under physiological conditions, there are detectable levels of BA and that testis can synthetized bile acids (Baptissart et al., 2014, 2016; Martinot et al., 2017b; Vega et al., 2015). In addition, previous reports demonstrate that steroids such as androsterone could be physiological ligands of FXRα (Wang et al., 2006). Thus, even if the endogenous/physiological ligand of FXRα has not been fully established in the testis, all these data clearly demonstrate that its signaling is active within the testis and can control either endocrine or exocrine function of the testis.

Overall, our data suggest that sensitivity to BPA exposure might be modulated by either concomitant exposures or genetic alterations. Here we demonstrate that FXRα may be a molecular "rheostat" that leads to variable thresholds for the development of male disorders in response to BPA encountered during embryonic/neonatal life.

EXPERIMENTAL PROCEDURES

Ethics Statement

The mouse study was conducted in compliance with the current regulations and standards approved by the Animal Care Committee (CE.72.12).

Human adult testes were obtained from prostate cancer patients who had no anti-androgen treatment and were not multi-organ donors (all donors considered, mean age 46.75 years, SEM ±4.65 years). The local ethics committee approved the protocol, and written informed consent was obtained from either donors or their next of kin (CCPPRB Rennes, authorization 05/39–566; Agence de la Biomédecine, authorization no. PFS09-015).

Testis Explant Assay

The testes obtained from patients or donors were placed at 4°C and processed immediately. Examination by transillumination showed that the testes displayed spermatogenesis (Roulet et al., 2006). Four 3-mm^3 testis explants were placed on a PET insert (BD Falcon, Lincoln Park, NJ, USA) at the interface of air in 1 mL of DMEM supplemented with antibiotics, 1 mM sodium pyruvate, 4 mM glutamine, 100 ng/mL of vitamin A, 200 ng/mL of vitamin E, 50 ng/mL of vitamin C, 10 μg/mL of insulin, 5 μg/mL of transferrin, and with 1 IU/mL hCG for culture, in 12-well plates. For the exposure experiments, the medium contained either 0.1% DMSO or chloroform (CHLORO) as a control, or BPA, S, or BS at different concentrations. When explants were treated by the BPA + S combination, the control corresponded to 0.1% of DMSO and 0.1% of CHLORO. Four wells (corresponding to the replicates for each independent experiment) were analyzed for each condition. Exposure to substances lasted 48 hr, with a total medium change at 24 hr. Medium was restored at −80°C. On the day of collection, three explants for each culture condition were collected at random, fixed in neutral-buffered 4% formalin, and embedded in paraffin. They were then sliced into 5.0-μm-thick sections, and stored at +4°C for subsequent immunostaining.

Animals

Fxrα$^{-/-}$ mice are described elsewhere (Baptissart et al., 2016; Martinot et al., 2017b, 2017a). The mice used in this study were housed in a temperature-controlled rooms with 12-hr light/dark cycles. Mice had *ad libitum* access to food and water. Pregnant female *Fxrα*$^{+/+}$ and *Fxrα*$^{-/-}$ were gavaged with vehicle (oil), BPA (50 mg/kg/day) (Sigma, 239658), S (5 mg/kg/day) (Sigma, S2424), or BS combined from PC6.5 until birth. Newborn male pups were injected subcutaneously with 5 μL of vehicle (oil), BPA (50 mg/kg/day), S (5 mg/kg/day), or BS combined for 5 days after birth with the same substances. Samples were collected at 10 days or 6 months.

Cell Studies

GC1-spg cells were used as described previously (Baptissart et al., 2014). Cells were treated for 7 hr with vehicle (DMSO/chloroform, 1:1,000, 10^{-8} M BPA [Sigma, 239658], 10^{-5} M S [Sigma, S2424]), or both molecules combined. Cells were then collected and mRNA extractions were performed. The data presented were obtained from four independent experiments with n = 3 per group per experiment.

Histology

After exposure, the testes were collected, fixed in 4% paraformaldehyde (PFA), embedded in paraffin, and 5-μm-thick sections were prepared and stained with H&E.

Immunohistochemistry

Paraffin sections of 4% PFA-fixed testis were sectioned at 5 μm. The sections were mounted on polysine glass slides, deparaffinized, rehydrated, treated for 25 min at 93°C to 98°C in citric buffer (0.01 M [pH 6]), rinsed in osmosed water (2 × 5 min) and washed (2 × 5 min) in Tris-buffered saline. Immunohistochemical analysis was conducted according to the manufacturer's recommendations as described elsewhere (Volle et al., 2009) for SOX9 antibodies (Millipore, AB5535), PLZF antibodies (Santa Cruz, H300 sc22839), PCNA antibodies (Santa Cruz, SC56), and H4-acetylated antibodies (Martinot et al., 2017a).

Endocrine Investigations

Plasmatic testosterone levels were measured using a commercial kit (Mybiosource, MBS494055).

RNA-Seq

The RNA-seq experiment was performed on testis of WT and *Fxrα*$^{-/-}$ mice at P10. Starting from RNA, all preparations were made using the IGBMC platform (Illkirch). The mRNA-seq libraries were sequenced (1 × 50 bases). Reads were mapped onto the mm10 assembly of the mouse genome using TopHat v.2.0.10 (Kim et al., 2013) and the Bowtie2 v.2.1.0 aligner (Langmead and Salzberg, 2012). Only uniquely aligned reads were retained for further analysis. Quantification of gene expression was performed using HTSeq v.0.5.4p3 (Anders et al., 2015) using gene annotations from Ensembl release 77. Read counts were normalized across libraries with the method proposed by Anders and Huber (2010). Comparison between FXRα$^{-/-}$ and WT samples was performed

using the method proposed by Love et al. (2014) implemented in the DESeq2 Bioconductor library (DESeq2 v.1.0.19). Resulting p values were adjusted for multiple testing using the method of Benjamini and Hochberg (1995). Dataset are given in supplemental table.

RNA-seq files are accessible on GEO: GSE119012.

Real-Time RT-PCR

RNA from mouse testis samples was isolated using Nucleospin RNA L (Macherey-Nagel, Hoerdt, France). cDNA was synthesized from total RNA with the MMLV reverse transcriptase and random hexamer primers (Promega, Charbonnières-les-Bains, France). The real-time PCR measurement of individual cDNA was performed using SYBR green dye (Master mix Plus for SYBR Assay, Eurogentec, Angers, France) to measure duplex DNA formation with the Eppendorf-Realplex system. For each experiment, standard curves were generated with pools of testis cDNA from animals with different genotypes and/or treatments. The results were analyzed using the ΔΔct method. Some of the primers were used in previous studies: *Plzf*, *Nanog*, *Lin28*, *Osp*, *Fshr*, *Fxrα* (Martinot et al., 2017a), *Stra8*, *Dmc1* (Martinot et al., 2017b), *Pxr* (44), *Sycp3* (Volle et al., 2007), *Cyp26b1*, *Shp*, *Esr1*, *Esr2* (Volle et al., 2009), Bsep, *G6Pase*, *Pepck*, *Apoa1* (Vega et al., 2014), *Dax1* (Baptissart et al., 2016), and *Dnmt3L* (fw GACGGAGCATT GAAGACATCT rv CACATAACCCTCCCTCAAACCA).

Statistics

Differences between vehicle group and groups treated with BPA, S, or BS were determined by ANOVA or two-way ANOVA. When significant effects of treatment or genotype or their interactions were obtained, multiple comparisons were made with Turkey's test. All numerical data are represented as means ± SEM. Significant difference was set at $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and one data file and can be found with this article online at https://doi.org/10.1016/j.stemcr.2018.08.018.

AUTHOR CONTRIBUTIONS

L.S., C.D.-L., B.R., L.T., C.D.-S., L.L., H.H., E.M., J.-P.S., S.M-G., and D.H.V. performed the experiments. L.S., C.D.-L., J.B., and D.H.V. designed the experiments. L.S., F.C., C.B., J.B., and D.H.V. wrote the manuscript.

ACKNOWLEDGMENTS

This study was conducted with the assistance of the "Anipath" Platform (GReD). We thank Sandrine Plantade, Khireddine Ouchen, and Philippe Mazuel for animal facilities. The research team headed by D.H.V. is funded by Inserm, CNRS, Clermont Université, Nouveau Chercheur Auvergne (no. R12087CC to D.H.V.), and Plan Cancer – Cancer-Environnement 2014–2019 (C14012CS). D.H.V.'s team received support from the French government IDEX-ISITE initiative 16-IDEX-0001 (CAP 20-25). Sequencing was performed by the IGBMC Microarray and Sequencing platform, a member of the "France Génomique" consortium (ANR-10-INBS-0009).

Received: October 18, 2017
Revised: August 27, 2018
Accepted: August 27, 2018
Published: September 20, 2018

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