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The health status alters the pituitary function and reproduction of mice in a Cxcr2-dependent manner

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Running title: Cxcr2 role in pituitary and reproduction - microbiota

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ABSTRACT

Microbiota and chronic infections can affect not only immune status, but also the overall physiology of animals. Here, we report that chronic infections dramatically modify the phenotype of *Cxcr2* knock-out (KO) mice, impairing in particular, their reproduction ability. We show that exposure of *Cxcr2* KO females to multiple types of chronic infections prevents their ability to cycle, reduces the development of the mammary gland and alters the morphology of the uterus due an impairment of ovary function. Mammary gland and ovary transplantation demonstrated that the hormonal contexture was playing a crucial role in this phenomenon. This was further evidenced by alterations to circulating levels of sex steroid and pituitary hormones. By analyzing at the molecular level the mechanisms of pituitary dysfunction, we showed that in the absence of *Cxcr2*, bystander infections affect leukocyte migration, adhesion and function, as well as ion transport, synaptic function behavior and reproduction pathways. Taken together, these data reveal that a chemokine receptor plays a direct role in pituitary function and reproduction in the context of chronic infections.

KEYWORDS

chemokine receptors, cytokines, microbiota, *Cxcr2*, pituitary

INTRODUCTION

Bystander chronic infections are common in rodent animal conventional facilities with a high prevalence of viruses such as mouse norovirus, parvovirus, mouse hepatitis virus, rotavirus, and bacteria such as helicobacter (Pritchett-Corning *et al*, 2009). Due to the possible deleterious effects of such infections, this has led to a recent trend of rethinking of the health status of animal facilities and the development of Specific and Opportunistic Pathogen Free (SOPF) - or of specific pathogen free (SPF) animal facilities to limit the influence of the environment on the phenotype of mice, especially in the case of immune or inflammatory studies. In SOPF conditions, mice are devoid of both pathogens and opportunistic infections; whereas in SPF conditions, they are devoid of pathogens only. However, there is quite a debate about using pathogen-free mice as animal models, since several reports have shown that mice exposed to bystander infections better recapitulate the human immune situation than mice housed in pathogen-free conditions (Beura *et al*, 2016) and that infections can affect the response to vaccination (Reese *et al*, 2016).

Moreover, it has also been shown that transplanting C57BL/6 embryos into domestic wild-type mice trapped in horse stables better recapitulate human immune response than laboratory animals, reinforcing the importance of microbiota (Rosshart *et al*, 2019). Such pathogen-free influences could also account for some of the difficulties in translating animal studies into treatments for patients. It remains that genetic alterations produced in mouse models frequently do not lead to the same phenotypes as those observed in humans with similar alterations. One relevant example is the response to infection (Cypowyj *et al*, 2012). Infections are transmitted through different generations of animals in particular during birth, but also during co-housing and breast feeding (McCafferty *et al*, 2013).

The role of microbiota is not only important in the context of immune studies (Hooper *et al*, 2012, Honda and Littman, 2016), but can also affect the outcome of different pathologies such as inflammatory bowel disease (Bloom *et al*, 2011), Crohn's disease (Cadwell *et al*, 2010), atherosclerosis (Wang *et al*, 2015), arthritis (Scher *et al*, 2013), asthma (Thorburn *et al*, 2015) or cancer (Roy and Trinchieri, 2017). Importantly, the genotype of mouse models does not contribute to the totality of phenotype observed and can be largely influenced by the various types of microbiota, with in some cases, a greater impact of the

microbiota than the genotype on the phenotype. This has led to the concept of "host gene plus microbe" or metagenome (Stappenbeck and Virgin, 2016). For these reasons, the use of SPF or SOPF husbandry can be viewed as an excellent way to normalize experiments and to limit the inter-individual or inter-housing variability and to improve the reproducibility of the results. However, factors other than microbiota can also deeply affect the phenotype of mouse models including husbandry conditions, such as temperature, light-dark cycles, diet, water, noise, hygrometry and handling of animals by care takers. Never-the-less, the microbiota is sometimes necessary to generate the phenotype. Indeed, a mouse model of Crohn's disease with mice harboring a mutation in *Atg16/1* gene, showed the expected phenotype in conventional conditions but not in SPF housing (Cadwell, *et al.*, 2010, Cadwell *et al.*, 2008). On the other hand, bystander infections can lead to a loss of a particular phenotype, such as in some models of diabetes (Okada *et al.*, 2010, Bach, 2002). This can be complicated further by the fact that the nature of microbiota can lead to different phenotypes, as exemplified in another model of Crohn's disease with mice deficient for *Nod2* (Ramanan *et al.*, 2014, Ramanan *et al.*, 2016).

Since one of the primary effects of bystander infections will be alterations to the immune system and the inflammation process (Tao and Reese, 2017), particular attention should be paid to pro-inflammatory cytokines and in particular chemokines. Chemokines are chemotactic cytokines of 60-100 amino acids that can be divided in four subtypes (CXC, CC, C or CX3C), based on the location of cysteines in N-terminus of the protein (Zlotnik and Yoshie, 2000). Chemokines are ligands of seven trans-membrane G α i protein-coupled receptors (GPCR), signaling in particular through the phosphatidylinositol-3 kinase (PI3K)/Akt, phospholipase C (PLC)/protein kinase C (PKC) and mitogen-activated protein kinase (MAPK)/p38, Ras/Erk and Janus kinase (JAK2)/signal transducer and activator of transcription (STAT3) pathways (Wang and Knaut, 2014).

Chemokines and their receptors play a major role in the trafficking of immune cells, notably during immune reaction or inflammatory events (McCully *et al.*, 2018), but their role is not restricted to immune processes, as they have been reported to be important in a number of other physiologic or pathologic events. These include angiogenesis (Strieter *et al.*, 2005), metabolism (Chavey *et al.*, 2009), chronic obstructive pulmonary disease (COPD) (Henrot *et al.*, 2019), neurodegenerative disease and cancer (Lazennec and Lam, 2016,

Lazennec and Richmond, 2010). Among chemokine receptors, *Cxcr2*, which is expressed in neutrophils and endothelial cells, appears essential in the control of angiogenesis, through the binding of ELR-motif containing chemokines (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8). ELR-motif chemokines harbor the tripeptide glutamic acid- leucine - arginine motif present in the N-terminal part of the protein (Strieter, Burdick, Gomperts, Belperio and Keane, 2005). *Cxcr2* regulates wound healing (Devalaraja *et al*, 2000), angiogenesis (Addison *et al*, 2000), multiple sclerosis (Liu *et al*, 2010), Alzheimer's disease (Tsai *et al*, 2002), atherosclerosis (Boisvert *et al*, 2000), respiratory diseases (Strieter *et al*, 2005), resistance to infections, (Cummings *et al*, 1999) and is involved in cancer (Lazennec and Richmond, 2010, Ali and Lazennec, 2007, Freund *et al*, 2003, Bieche *et al*, 2007). *Cxcr2* knock-out animals exhibit splenomegaly due to an increased number of metamyelocytes and neutrophils, and impairment in the recruitment of neutrophils during acute inflammatory conditions (Cacalano *et al*, 1994).

Here, we report that the action of microbiota on mouse phenotype is dependent on the absence of *Cxcr2* protein. In the absence of *Cxcr2*, mice are clearly affected by the presence of pathogens. However, in the absence of pathogens, *Cxcr2* knock-out (KO) mice display a similar external phenotype to that of Wildtype (WT) mice in terms of their ability to reproduce and their gross appearance (Cacalano, *et al.*, 1994, Broxmeyer *et al*, 1996). By contrast, in conditions of bystander infections, *Cxcr2* null mice exhibit an impaired reproductive ability and reduced development of reproductive organs. Using mammary gland and ovary transplant experiments, we show that reproductive function can be restored to *Cxcr2* KO mice in a WT context, despite the presence of pathogens. We also show that the absence of *Cxcr2*, not only leads to susceptibility to infection, it additionally leads to reproductive defects due to major impairment of pituitary function controlling the production of pituitary hormones. This study therefore reveals a novel role for the chemokine receptor *Cxcr2* in pituitary physiology, that has been discovered in the context of microbiota infections. This has never been reported for any chemokine receptor.

RESULTS

We have been working for a long time on *Cxcr2* ligands (Freund, *et al.*, 2003, Bieche, *et al.*, 2007, Freund *et al.*, 2004) and we wished to use *Cxcr2* KO animals to analyze its role *in vivo*. Our study started with the serendipitous finding that *Cxcr2* KO animals had distinct breeding abilities in conventional or SOPF animal facilities. In order to evaluate the possible action of microbiota mouse phenotype in the context of *Cxcr2* deficiency, mice were housed either in a SOPF animal facility in sterile conditions or in a conventional animal facility with possible bystander infections. In SOPF conditions, *Cxcr2* KO animals displayed the same breeding ability as WT animals, confirming prior work of Cacalano (Cacalano, *et al.*, 1994) (Fig. 1A). On the contrary, after transfer of SOPF animals in conventional conditions, we observed that after several generations in conventional conditions, *Cxcr2* KO animals lost their breeding ability. Both males and females were infertile (Fig. 1B), even when mated with WT animals. Of particular note, the product of such mating, *Cxcr2* heterozygous animals, were much less affected by these conditions and could breed nearly normally (although with a delayed time for successful breeding) and generate *Cxcr2* KO animals (data not shown). Moreover, *Cxcr2* KO females of conventional conditions exhibited a smaller weight than WT animals (Fig. 1D), which was not the case in SOPF conditions (Fig. 1C). Screening of bystander infections of animals housed in conventional conditions showed the presence of mouse norovirus, helicobacter and Entamoeba SP (supplemental Fig. 1). We hypothesized that these infections were responsible for the observed phenotype as rederivating conventional animals to remove pathogens led to animals with full reproduction ability. Moreover, this combination of pathogens was not specific for the phenotype observed, as housing *Cxcr2* KO animals in other conventional facilities with another set of pathogens (including mouse norovirus, mouse hepatitis virus, other strains of helicobacter or pinworms) led to the same results (supplemental Fig. 1).

To understand why *Cxcr2* KO animals were infertile in conventional conditions, we decided to focus on females. We first performed vaginal smears of WT and KO animals of conventional conditions (Fig. 2A). This showed that WT animals displayed a classical cycling with proestrus, estrus, metestrus and diestrus. On the other hand, KO animals displayed mixed populations of cells, with no real homology to any steps of estrus

cycling, suggesting that the mice were not cycling. In SOPF conditions, WT and KO animals displayed a normal estrus cycle (Supplemental Fig. 2). To assess the functionality of the ovary, we analyzed the ovaries of WT and KO animals from conventional or SOPF conditions. We observed that in SOPF conditions, both WT and KO animals displayed a normal histology of the ovary, with all stages of follicles maturation as well as the presence of multiple corpora lutea, suggesting that the mice were able to ovulate (Fig. 2B, upper panel). On the contrary, in conventional conditions, while the ovary of the WT animals appeared to have completely normal histologic appearance, ovaries from KO animals displayed a large number of follicles in all stages of development including atresia, but did not exhibit any corpus luteum, suggesting that KO *Cxcr2* mice could not ovulate (Fig. 2B, lower panel).

We next looked at other reproductive organs including uterus and mammary gland. The uterus of WT and KO animals in SOPF conditions appeared normal and of similar size (Fig. 3A, upper panel). In conventional conditions, the uterus of KO animals was smaller in diameter compared to WT animals and was in a rest status, whereas WT uterus was cycling (Fig. 3A lower panel). Maximum uterine thickness and the external uterine diameter were approximately 4-fold reduced in KO animals compared to WT animals (Supplemental Fig. 3A). Whereas uteri of WT mice had well defined layers, uterine layers were less discernable in KO animals, appearing compressed and very cellular. KO mice also showed a loss of the normal convoluted appearance of the uterine luminal epithelium, assuming a more linear profile. The endometrium thickness, the luminal epithelium thickness as well as the number of glandular lumen were also decreased in KO animals, suggesting a non cycling uterus (Supplemental Fig. 3B).

We also observed an altered morphology of the mammary gland in KO animals in conventional conditions. Whole mount experiments showed a complete branching in WT animals whereas the mammary gland of KO animals displayed a rudimentary branching (Fig. 3B). Mammary glands of KO appeared to have a significant reduction of the numbers of glandular (ductal) profiles (Supplemental Fig. 3C, lower panel). Epithelial cells lining ducts in KO mice often appeared haphazardly arranged and the mammary gland than its WT counterpart. The situation appeared different in the mammary gland of SOPF animals with a similar branching in WT and KO animals (Supplemental Fig. 3C, upper panel).

In order to understand the reasons for the reproductive defects in KO animals housed in conventional conditions, we decided to first compare the transcriptomic profiles of the mammary gland of WT and KO animals in SOPF with those in conventional conditions by RNAseq. Principal component analysis (PCA) of RNAseq showed that mammary glands of WT and KO animals were close to each other, whereas the one of WT and KO conventional animals were more widely distributed (Supplemental Fig. 4). We observed that the transcriptome of the mammary gland of KO animals was much more altered in conventional, as compared to SOPF conditions, with approximately 10-fold more genes up-regulated in WT animals versus KO animals (Fig. 4A). Among the genes up-regulated in the mammary gland of WT compared to KO animals, only 12 were common between conventional and SOPF conditions (Fig. 4B), and only 4 were common for down-regulated genes. Gene ontology (GO) analysis showed that the common down-regulated genes were essentially related to leukocyte chemotaxis and migration, and host defense (Fig. 4C). In SOPF conditions, the genes down-regulated in the mammary gland of KO animals were essentially those linked to muscle development and differentiation (Fig. 4D and Table 1), whereas the genes up-regulated in KO mice were related to granulocyte migration and leukocyte aggregation/adhesion (Fig. 4E and Table 2). We next focused on the major alterations of the transcriptome of the mammary gland of KO animals in conventional conditions. Gene ontology analysis showed that most of the genes down-regulated in the mammary gland of KO animals were related to three major biological processes (Table 3): mammary gland development and differentiation (Fig. 5A), epithelial cell proliferation (Fig. 5B) and Wnt signaling (Fig. 5C). On the other hand, genes up-regulated in the mammary gland of KO animals in conventional conditions (Table 4) were involved in leukocyte migration (Fig. 5D) and muscle function (Fig. 5E). The RNAseq data were validated by real time PCR on a subset of representative genes of the different GO identified or known to play a role in mammary gland physiology. We observed a common up-regulation of S100a8, S100a9, Mmp8 and Ngp genes related to chemotaxis in the mammary gland of KO animals in conventional and SOPF conditions (Fig. 6). On the other hand, Areg, lactoferrin, CXCL15, Elf5, Sox10, Ido1, Wnt2, Prlr, Krt15 and Gata3 were down-regulated only in the mammary gland of KO animals in conventional conditions (Fig. 6). Of particular note, Areg, Lactoferrin, Wnt2, Prlr, Elf5, and Gata3 are genes known to be critical for development of the mammary

gland according to the studies performed with KO animals for these genes (Kelly *et al*, 2002, Lueteteke *et al*, 1999, Kouros-Mehr *et al*, 2006, Zhou *et al*, 2005, Watson and Khaled, 2008).

We also analyzed the differences in the ovary of WT and KO animals in conventional conditions, by looking at some key genes known to play a role in ovary function. We report a decrease in the expression of *Akr1c18*, *Cyp19*, *Hsd3b2*, *Prlr* and lactoferrin genes in the ovary of KO animals, whereas AR expression was strongly induced (Fig. 7A). *Akr1c18* encodes 20 α -hydroxysteroid dehydrogenase, a progesterone metabolizing enzyme (Piekorz *et al*, 2005). *Cyp19* or estrogen synthase is an aromatase of the P450 family involved in, in particular, the aromatization of androgens to estrogens (Rosenfeld *et al*, 2001). *Hsd3b2* encodes hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2, which is involved in the conversion of 5-ene-3 β -hydroxysteroids to 4-ene-3-ketosteroid, an essential step in the biosynthesis of progesterone and estrogens in the ovary (Payne *et al*, 1995). Interestingly, many of these enzymes are regulated by prolactin, and prolactin receptor (*Prlr*) is critical (Stocco *et al*, 2007, Bachelot and Binart, 2005). Androgen receptor (*Ar*) also plays a critical role in ovary function, and *Ar* KO leads to premature ovarian failure (Shiina *et al*, 2006, Walters, 2015). The alteration of these key regulatory genes in the ovary suggested to us, a possible impairment of hormone production. We thus measured progesterone and estradiol serum levels in WT and KO animals in conventional conditions. In agreement with the absence of corpus luteum in KO ovaries, we observed a decrease in progesterone levels relative to WT (Fig. 7B). On the other hand, estradiol levels were increased.

This led us to hypothesize that an alteration of the hormonal context could explain the reproductive defects observed in KO animals housed under conventional conditions. To test this, we performed ovary transplantation experiments (Fig. 7C). WT mice were ovariectomized and a WT or KO ovary was re-implanted within the oviduct bursa (Behringer, 2017). We observed that both WT and KO transplanted ovaries were able to display a normal phenotype with the presence of corpora lutea (Fig. 7C). Moreover, when transplanted females were bred, they were able to give birth (Fig. 7C). To confirm the role of the hormonal environment in the KO defects, we also performed mammary gland transplantation (Fig. 7D). The mammary fat pads of young WT female mice were cleared of all epithelial structures and either re-

implanted with WT or KO mammary gland fragments or left untreated. Control mammary glands without transplant did not develop any ductal branching, whereas both WT and KO transplants could fully restore a functional mammary gland (Fig. 7D). Together, these data suggest that the hormonal environment of WT animals is sufficient to enable the KO ovary and mammary gland to be functional. As steroid hormone production is controlled by the pituitary, we assessed the serum levels of the pituitary hormones FSH, LH, PRL and GH in WT and KO animals in conventional conditions. We report that the four pituitary hormones tested displayed a clear decrease in KO animals (Fig. 8A), suggesting major defects in the pituitary function of KO animals housed under conventional conditions. We did not measure the hormone levels of transplanted animals (Fig. 7C), as these mice were used for breeding and could not be compared with virgin animals.

To understand at the molecular level, the reasons for the pituitary dysfunction in KO animals, we performed an RNAseq analysis of pituitary glands from WT and KO animals in SOPF and conventional conditions (Fig. 8B). Strikingly, very little difference was observed between the pituitaries of WT and KO animals in SOPF conditions. On the other hand, more than 850 genes were either up-regulated or down-regulated in the pituitary of KO animals in conventional conditions (Fig. 8B). Principal component analysis (PCA) of RNAseq showed that pituitary of WT and KO animals were close to each other, whereas the one of WT and KO conventional animals were very different (Supplemental Fig. 4). According to gene ontology analysis, the up-regulated pathways in the KO pituitaries were related to immune cell activation (in particular lymphocyte), leukocyte adhesion, and neutrophil motility and extravasation (Fig. 8C and Table 5). On the other hand, down-regulated genes involving biological processes included those involved in ion transport and synaptic function (Fig. 8D and Table 6), as well as control of ovarian function (Fig. 8E and Table 6). To validate these data, we analyzed the expression of a set of genes representative of the different GO mentioned above by real-time PCR on a larger number of animals (Fig. 9). *Elane*, *S100a8*, *S100a9*, *Mpo*, *Ngp*, *MMP8*, *Ltf* and *Serpina3an* were strongly up-regulated in the pituitary of KO animals in conventional conditions and modestly or not regulated at all, in the pituitary of KO animals in SOPF conditions. *Elane*, *S100a8*, *S100a9* are involved in migration, adhesion, and immune response. *Mpo*, *Ngp*,

Ltf and *Serpina3an* are contributing to migration and immune response. In contrast, *Prl*, *Crhbp*, *Akr1c14*, *Vip* and *Rln* were all down-regulated in the pituitary of conventionally housed KO animals but not in SOPF conditions (Fig. 9). *Prl*, *Crhbp* and *Vip* are involved in ion transport, behavior and reproduction. *Rln* and *Crhbp* play a role in ion transport, synapse function and behavior.

DISCUSSION

In this study, we investigated the effects of bystander infections on the physiologic role of the chemokine receptor *Cxcr2* using *Cxcr2*-null mice. Our results have demonstrated that when exposed to common infections found in animal facilities, *Cxcr2* knock-out mice, but not WT mice, exhibit reproductive defects. Of particular note, CXCR2 ligand levels were not altered in the mammary gland and the pituitary gland of WT animals in conventional conditions compared to SOPF conditions, according to RNAseq analysis. Homozygous males and females were both sub-fertile and females displayed alterations to their secondary sex organs. The first observation accounting for this sub-fertility was that female *Cxcr2* KO mice housed in conventional conditions were not able to cycle normally. None of the characteristic phases of the estrous cycle could be identified in these animals by vaginal smear, with mixed populations of vaginal cells suggesting a defect in ovarian function. Microscopic observation of ovaries from *Cxcr2* KO animals housed in conventional conditions showed reductions of the size of the ovary and an absence of corpora lutea, whereas in SOPF conditions, both WT and *Cxcr2* KO ovaries showed normal histology with all stages of follicle development and corpora lutea present. Interestingly, a number of genes involved in ovarian function and production of steroid hormones (*Akr1c18*, *Cyp19*, *Hsd3b2*, *Prlr*, *lactoferrin*, *Ar*) were down-regulated in the ovary of *Cxcr2* KO animals in conventional conditions. *Akr1c18* (20alpha-hydroxysteroid dehydrogenase), which catabolizes progesterone into 20alpha-dihydroprogesterone (inactive steroid) is necessary for the maintenance of pregnancy (Choi *et al*, 2008) and knock out of this gene leads to longer duration of estrous cycle and a reduced number of pups (Ishida *et al*, 2007). Knocking down *Cyp19* (Aromatase P450) has been shown to lead to mice lacking corpus luteum in ovary, accompanied by total infertility (Toda *et al*, 2001). *Hsd3b2* (hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2) plays a crucial role in the biosynthesis of many steroids (Chapman *et al*, 2005). *Prlr* (prolactin receptor) KO animals are completely infertile with irregular estrous cycles (Horseman *et al*, 1997). *Lactotransferrin*, present in the reproductive tracts of rodents, can regulate secretory function and plays a role in fertilization (Yanaihara *et al*, 2007). *Ar* (androgen receptor) KO mice show a marked reduction in follicular maturation at maturity, with fewer corpora lutea in their ovaries (Hu *et al*, 2004). Not surprisingly

then, morphometry, using multiple histological sections in the present study, identified alterations to the uteri of *Cxcr2* KO animals housed conventionally, with global reductions in uterine size, as well as a decrease in endometrial thickness and the number of glands present, suggesting uterus in arrest not cycling under hormonal stimulation.

The mammary glands of *Cxcr2* KO females housed in conventional facilities were also minimally developed, harboring a phenotype close to that of juvenile animals with a rudimentary duct branching. In contrast, in SOPF conditions, mammary gland development appeared similar to that of WT animals. Interestingly, even in aged animals, no further development of the mammary gland could be observed in *Cxcr2* KO females housed in conventional facilities (data not shown). At the molecular level, the mammary gland of *Cxcr2* KO animals showed a clear down-regulation of the expression of genes involved in mammary gland development and differentiation, epithelial cell proliferation, wound healing and Wnt signaling (including *lactoferrin*, *Gata3*, *cyclin D1*, *ErbB2*, *Elf5*, *Epcam*, *Prlr*, *Krt15*, *Epcam*, *Wnt4*, *Wnt5b*). For a number of the down-regulated genes, invalidation studies in mice have shown that these genes (*Prlr*, *cyclin D1*, *ErbB2*, *Elf5*) are crucial for mammary gland development (Zhou, *et al.*, 2005, Bole-Feysot *et al.*, 1998, Howlin *et al.*, 2006). The Wnt pathway plays a pivotal role in orchestrating proper mammary gland development and maintenance (Jarde and Dale, 2012), and this pathway was impaired in *Cxcr2* KO animals. The Wnt pathway is a major paracrine mediator of hormonal action, through Wnt4 and Areg pathways in particular (Ciarloni *et al.*, 2007, Brisken *et al.*, 2000), both of which are down-regulated in the mammary glands of *Cxcr2* KO animals housed conventionally. The general scheme of Wnt action in the mammary gland is based on the activation of estrogen receptor alpha (ER α)-positive luminal cells, which in turn are stimulated by steroid hormones to release Wnt ligands that will act directly or indirectly on the myoepithelial compartment (Jarde and Dale, 2012).

The collection of evidence gathered herein, for possible hormonal perturbations affecting *Cxcr2* KO animals housed in conventional conditions; including mammary, ovary and uterus histologic changes and molecular defects, led us to evaluate steroid hormone levels in these animals. We observed in particular, a decrease

in serum progesterone levels, that could reflect the absence of corpora lutea in the ovary of these animals, the main site of production of progesterone.

To demonstrate the role of the hormonal environment in the phenotype observed for *Cxcr2* KO animals in conventional conditions, we decided to perform ovarian transplantation experiments. By transplanting the ovary of *Cxcr2* KO animals into the ovarian bursa of ovariectomized WT animals, we observed that KO ovaries could display a normal histology, close to WT ovaries, with the presence of corpora lutea, a sign of successive ovulations. Moreover, the transplanted animals had their fertility restored and were able to give birth to viable mice. Similarly, transplantation of the mammary glands of *Cxcr2* KO animals into a WT context also led to the development of normal gland development with correct ductal branching. Taken together, this confirms that the hormonal context of WT animals is sufficient to restore a correct function and development of the *Cxcr2* KO ovary and mammary gland. As the function of the ovary, the uterus and the mammary gland are tightly controlled by steroid hormones and at a higher level, by pituitary hormones, we measured the circulating levels of FSH, LH, GH and PRL. The levels of these four pituitary hormones were markedly decreased in *Cxcr2* KO animals housed in conventional conditions compared to WT animals, suggesting an alteration to pituitary function. Treating the KO animals with FSH and LH to restore fertility could be an interesting issue, but it is likely that the timing will be critical and difficult to assess due to the lack of cycling of these animals.

Transcriptomic and gene ontology analysis of the pituitary of WT and *Cxcr2* KO animals revealed as expected, a down-regulation of genes involved in the control of circadian rhythm, ovulation control, and gonad development; which could account for dysregulation of pituitary hormones. This includes genes such as *Esr1* (estrogen receptor alpha), *Pgr* (Progesterone receptor), *RMB4* (required for the translational activation of PER1 mRNA in response to circadian clock) (Markus and Morris, 2009), *Crebbp* (CREB-Binding Protein, involved in circadian clock) (Rexach *et al*, 2012), *Foxo3* (a regulator of circadian clock) (Chaves *et al*, 2014). Moreover, one could expect behavior alterations of *Cxcr2* KO animals based on the down-regulation of a number of genes controlling behavior, including for instance *Crhbp* (Corticotropin Releasing Hormone Binding Protein) (Ketchesin *et al*, 2017), *Oprk1* (opioid receptor, kappa 1) (Loh *et al*, 2017) or *Vip*

(vasoactive intestinal polypeptide) (Hill, 2007). The dysfunction of the *Cxcr2* KO pituitary involves presumably defects in synapse function, as well as ion transport. Indeed, we report a down-regulation of a number of genes involved in calcium, sodium and potassium transport or in synaptic transmission such as *Vip*, *Oprk1*, *Kcnb2* (potassium voltage gated channel, *Shab*-related subfamily, member 2) or *Trpc6* (transient receptor potential cation channel, subfamily C, member 6), *Cacna1g* (calcium channel, voltage-dependent, T type, alpha 1G subunit). Synaptic alteration includes anterograde trans-synaptic signaling, long-term synaptic potentiation and synaptic plasticity and could have major effects on neuronal connections. In addition to down-regulation of the pathways mentioned above, other pathways appeared up-regulated in the pituitary of *Cxcr2* KO animals. This includes in particular aggregation and adhesion of leukocytes as well as up-regulation of chemotaxis, extravasation, tethering or rolling and also leukocyte activation, proliferation and differentiation. When comparing our RNAseq results with signatures for B lymphocytes, T lymphocytes, macrophages and neutrophils from Nirmal et al. (Nirmal et al, 2018), it appears, that there is a T lymphocyte enrichment (21.%) and to a lesser extent of B lymphocytes (13.5%), macrophages (12.8%) and neutrophils (6.4%) (Supplemental table 2). This possible infiltration of T and B lymphocytes in the pituitary could be reminiscent of Autoimmune Hypophysitis (AH), which is an inflammatory disease of the pituitary gland, and particularly in relation to lymphocytic hypophysitis (Bellastella et al, 2016). AH can lead to atrophy of the pituitary and to hypo-pituitarism, which is in agreement with what we observed for *Cxcr2* KO animals in conventional conditions. Functional disturbances observed in humans with AH include headaches, visual disturbances, hypothyroidism, hypogonadism and an inability to lactate (Caturegli et al, 2005).

Whereas multiple links between gut microbiota and the hypothalamic-pituitary axis have been reported (Appleton, 2018), our results constitute the very first evidence for the involvement of a chemokine receptor, *Cxcr2*, in the control of pituitary function and the reproductive system and that this phenomenon is dependent on microbiota (Fig. 10). Although there have to this time been no reports of any action of *Cxcr2* in pituitary function, *Cxcr2* has been shown to be important for the positioning of oligodendrocyte precursors in the spinal cord (Tsai, et al., 2002) and in myelin repair (Liu, et al., 2010), but overall the effects

of *Cxcr2* in central nervous system are poorly understood. On the other hand, a few reports have suggested a potential role for *Cxcr2* in controlling the effects of microbiota. Indeed, it was shown that microbiota promotes the recruitment of *Cxcr2*-positive neutrophils that can protect from amebic colitis (Watanabe *et al*, 2017). Finally, so far, no direct evidence of a role of *Cxcr2* in the control of reproduction has been reported. Some reports suggest that *Cxcr2* might contribute to the development of preeclampsia (Wu *et al*, 2016, Chen *et al*, 2019) and in the control pregnancy tolerance through neutrophils (Kang *et al*, 2016). Additionally, loss of *Cxcr2* ligand has been shown to result in premature senescence in placenta-derived mesenchymal stem cells (Li *et al*, 2017).

In summary, our results highlight a unique unexplored role for *Cxcr2* in pituitary and reproductive function that is dependent on chronic infections (summarized in Fig. 10). These findings reinforce an imperative; that in order to be translationally relevant, the use of animal models to understand disease mechanisms must take in account animal husbandry conditions and disease phenotyping requiring the absence or presence of chronic infections.

LEGENDS TO FIGURES

Fig.1: Husbandry in conventional housing conditions alters the reproduction of *Cxcr2* KO animals

A. Number of animals/litter issued in the breeding of WT animals or *Cxcr2* KO animals in SOPF conditions. The data represent the mean \pm SEM of at least 10 matings. (Mann-Whitney test, NS: non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). **B.** Same breeding experiment in conventional conditions with bystander infections. The results represent the mean \pm SEM of at least 8 animals. **C.** Weight of 12 week females in SOPF conditions. **D.** Weight of 12w females in conventional conditions. The data represent the mean \pm SEM of at least 8 animals. (Mann-Whitney test, **** $p < 0.0001$).

Fig. 2: *Cxcr2* KO animals in conventional housing conditions exhibit cycle defects and altered ovary morphology

A. Representative brightfield microscopic images (40x) for Giemsa stained vaginal smears from different estrous cycle stages of WT and KO animals housed in conventional conditions. For WT animals, day 1: Proestrus (mostly nucleated epithelial cells), day 2: estrus (cornified epithelial cells), day 3: metestrus (cornified epithelial cells with leukocytes), day 4: diestrus (mostly leukocytes). For KO animals, the content of vaginal smears, no clear state of estrus cycle could be determined. Scale bars: 200 μ m. **B.** Histology of the ovary of WT and KO animals in SOPF (upper part) or conventional conditions (lower part). Representative images of hematoxylin-eosin stained ovaries at a 5x magnification are shown here. Stars indicate the presence of corpus lutea. Scale bars: 500 μ m.

Fig. 3: The uterus and mammary gland of *Cxcr2* KO animals show defects in conventional conditions

A. Histology of the uterus of WT and KO animals in SOPF (upper part) or conventional conditions (lower part). Representative images of hematoxylin-eosin stained uteri at a 5x magnification are shown here. Scale bars: 500 μ m. **B.** Whole mount of mammary glands from 13 week WT and KO animals in conventional conditions. Scale bars: 5mm (left panel) or 1.3mm (right panel).

Fig. 4: Differential gene expression in WT and KO mammary glands is more pronounced in conventional conditions

A. Left panel: the Volcano plots show the global changes in RNA expression patterns for WT versus KO mammary glands in SOPF or conventional conditions. Data represent analysis of cpm estimates with a log of fold change of more than 1.5 fold change and $p < 0.05$ of 3 animals per group. Right panel: Number of differentially regulated genes for the same analysis. **B.** Left panel: Venn diagram representing the common genes up-regulated or down-regulated in the mammary gland of WT compared to KO animals in conventional versus SOPF conditions. Right panel: list of common genes. **C.** Gene Ontology analysis of Biological Process for the common genes regulated in KO animals in conventional and SOPF conditions. **D.** Gene Ontology analysis of Biological Process of down-regulated genes in KO mammary glands of SOPF are mostly related to muscle function. Black arrows mean "is a". Blue arrows mean is "part of". **E.** Gene Ontology analysis of Biological Process of up-regulated genes in KO mammary glands of SOPF are mostly related to granulocyte chemotaxis (left panel) and to neutrophil aggregation (right panel).

Fig. 5: *Cxcr2* KO affects mammary gland function in conventional conditions

A, B, C. Gene Ontology analysis of down-regulated genes in the mammary gland of KO animals. Black arrows mean "is a". Blue arrows mean is "part of". Green arrow means "positively regulates". Red arrow means "negatively regulates". Yellow arrow means "regulates" **A.** Biological Process related to mammary gland function. **B.** Similar analysis as in B in terms of cell proliferation. **C.** Similar analysis as in B in terms of Wnt signaling. **D & E.** Gene Ontology analysis of up-regulated genes in the mammary gland of KO animals. **D.** Biological processes related to chemotaxis. **E.** Biological processes related to muscle function.

Fig. 6: *Cxcr2* KO affects mammary gland transcriptome in conventional conditions

Measure of RNA levels by real-time PCR of a set of genes in the mammary gland of WT and KO animals in conventional or SOPF conditions. Results represent the mean the mean \pm SEM of at least 12 animals (Mann-Whitney test, NS: non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Fig. 7: *Cxcr2* KO display an alteration of hormonal function in conventional conditions. **A.** Measure of RNA levels of a set of genes in the ovary of WT and KO animals in conventional conditions by real-time PCR. Results represent the mean the mean \pm SEM of at least 7 animals (Mann-Whitney test, NS: non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). **B.** Serum levels of estradiol and progesterone in WT and KO mice in conventional conditions. Box and whiskers represent the min and max of at least 10 animals (Mann-Whitney test, NS: non-significant, * $p < 0.05$, ** $p < 0.01$). **C.** Left panel: strategy of ovary transplantation. A *Cxcr2*-WT mouse was ovariectomized and re-implanted with either WT or KO ovary from a conventional facility. Once the graft was established, females were bred with WT males to evaluate their fertility. Right panel: Histology of the transplanted *Cxcr2* WT and *Cxcr2* KO ovaries. Representative images of hematoxylin-eosin stained ovaries at a 5x magnification are shown here. Scale bars: 500 μ m. The % of successful breeding of transplanted recipient females is indicated. Fisher exact test shows no difference between WT and KO successful breeding ($p = 0.5147$). The number of corpora lutea in WT or KO transplanted ovaries is also presented and shows no statistical difference (Mann-Whitney test, NS). **D.** Left panel: strategy of mammary gland transplantation. The mammary gland fat pads of *Cxcr2*-WT mice were cleared and transplanted with either *Cxcr2*-WT or *Cxcr2* KO mammary gland. Right panel: Whole mounts of mammary glands of recipient mice after no transplantation or transplantation with WT or KO mammary glands. Scale bars: 5mm (left panel) or 1.3mm (right panel).

Fig. 8: Circulating pituitary hormones and transcriptome in the pituitary of KO animals are drastically affected in conventional housing conditions

A. Serum levels of pituitary hormones FSH, LH, PRL and GH. Results represent the mean the mean \pm SEM of at least 14 animals (Mann-Whitney test, NS: non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). **B.** Left panel: the Volcano plots show the global changes in RNA expression patterns for WT versus KO pituitary in SOPF or conventional conditions. Data represent analysis of cpm estimates with a log of fold change of more than 1.5 fold and $p < 0.05$ of 3 animals per group. Right panel: Number of

differentially regulated genes for the same analysis. **C.** Gene Ontology analysis of up-regulated genes in the pituitary of KO animals in conventional conditions. **D.** Gene Ontology analysis of down-regulated genes in the pituitary of KO animals in conventional conditions linked to synapse function and ion transport. **E.** Gene Ontology analysis of down-regulated genes in the pituitary of *Cxcr2* KO animals in conventional conditions linked to reproduction.

Fig. 9: Gene expression in the pituitary of KO animals in conventional housing conditions

Measure of RNA levels by real-time PCR of a set of genes in the pituitary of WT and *Cxcr2*-KO animals in conventional or SOPF housing conditions. Results represent the mean the mean \pm SEM of at least 12 animals. Kruskal-Wallis test was used followed by Dunn's multiple comparisons test to compare all groups between each other.

Fig. 10. General scheme of events occurring in *Cxcr2* KO mice under pathogen infection.

Infection of *Cxcr2* KO animals by microbiota (viruses or bacteria) leads to an alteration of the pituitary function, with a defect in the production of pituitary hormones such as LH, FSH and Prl. Low levels of these hormones impairs the function of the ovary (with an absence of ovulation) and production of progesterone. In turn, both uterus anatomy and the development of the mammary gland are affected.

Table 1. Most enriched pathways for genes down-regulated in mammary gland of KO versus WT SOPF animals

Table 2. Most enriched pathways for genes up-regulated in mammary gland of KO versus WT SOPF animals

Table 3. Most enriched pathways for genes down-regulated in mammary gland of KO versus WT conventional animals

Table 4. Most enriched pathways for genes up-regulated in mammary gland of KO versus WT conventional animals

Table 5. Most enriched pathways for genes up-regulated in pituitary gland of Cxcr2-KO versus Cxcr2-WT conventional animals

Table 6. Most enriched pathways for genes down-regulated in pituitary of KO versus WT conventional animals

EXPERIMENTAL PROCEDURES

Animal Models and housing

All animal experiments conform to our animal protocols that were reviewed and approved by the Institutional Animal Care and Use Committee. *Cxcr2*^{-/-} mice (Cacalano, *et al.*, 1994) obtained from the Jackson Laboratory were in BALB/c genetic background as well as their littermate control (WT) mice. Mice were either housed in a SOPF facility or transferred to a conventional animal facility. To favor the acquisition of microbiota from conventional facility to the SOPF animals which were transferred to conventional conditions, the bedding of conventional animals was added to the one of transferred SOPF animals during the first weeks. The phenotype of the animals housed in the conventional facility was first analyzed following at least one or two generations of breeding to enable the establishment of bystander infections in all animals. Due to the reduced fertility of *Cxcr2*^{-/-} animals in conventional conditions, homozygous animals were obtained by crossing heterozygous animals.

Pathogen screening

The presence of potential infections was monitored by Charles River Research Animal Diagnostic Services on whole animals or body swabs, oral swabs and feces of the animals according to PRIA instructions. The main bystander infections observed are shown in Supplementary Figure 1.

Analysis of estrus cycle

Vaginal smears of WT and KO mice were performed in NaCl 9/1000, fixed in methanol and then stained with Giemsa dye. Slides were scanned with a nanozoomer (Hamamatsu).

Whole mounts of mammary glands

Mammary glands were spread on glass slides, fixed in Carnoy's fixative for 4 hours. After rehydration, the glands were stained with carmine alum stain overnight. Glands were then cleared in xylene for several weeks to eliminate the fat.

RNA extraction and reverse transcriptase, quantitative PCR

Total RNA was isolated using TRIzol reagent (ThermoFisher), as described by the manufacturer. Reverse transcription was performed with 1µg of total RNA using random primers and with M-MLV enzyme (ThermoFisher). Real time quantitative PCR was realized with SYBR green Master Mix (Roche), on a Light Cycler 480 instrument (Roche) as previously described [23]. Ribosomal protein S9 (rS9) and GAPDH were used as an internal control. The sequence of the primers used in this study is indicated in Supplemental Table 1. Results are expressed as N-fold differences in target gene expression relative to the internal control gene and termed "mRNA expression", were determined as $\text{mRNA expression} = 2^{-\Delta\text{Ct}_{\text{sample}}}$, where the ΔCt value of the sample was determined by subtracting the Ct value of the target gene from the Ct value of the average of the internal control genes. Target genes were considered to be non-detectable when the Ct value was above 35.

RNA-seq data processing

RNA integrity and quality was verified using RNA ScreenTape kit and Tapestation 2200 apparatus from AGILENT. cDNA libraries were synthesized using SureSelect-Strand-Specific mRNA library prep (Agilent). Library quality was checked on Tapestation 2200 apparatus from AGILENT with DNA 1000 ScreenTape. Samples were sequenced on NextSeq 500 (Illumina) with an average sequencing depth of 30 millions of paired-end reads. Length of the reads was 75 bp. Each 12 Plex Samples was sequenced on one HighOutput FlowCell (2*400Millions of 75bases reads). Raw sequencing data was quality-controlled with the FastQC program. Low quality reads were trimmed or removed using Trimmomatic (minimum length: 40 bp). Reads were aligned to the mouse reference genome (mm10 build) with the TopHat2 tool (option for no

multihits). Mapping results were quality-checked using RNA-SeQC. Gene counts were obtained by read counting software featureCounts. Normalization and differential analysis were performed with the DESeq2 package with Benjamini-Hochberg FDR multiple testing correction ($p \text{ corr} < 0.05$; 1.5-fold or higher change) comparing WT and KO animals. Clustering analysis was performed with Genesis software (Sturn *et al*, 2002). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al*, 2002) and are accessible through GEO Series accession number GSE144231 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE144231>).

Hormone assessment

Whole sera were used to measure the levels of estradiol and progesterone by Elisa with specific kits from Abnova. The levels of LH, FSH, GH and PRL were assessed by luminex with Mouse Pituitary Magnetic Bead Panel (Merck Millipore).

Ovary and mammary transplantations

For ovary transplantations, WT or KO ovaries were obtained from 10 week-old donor mice. WT recipients were anesthetized with isoflurane and ovariectomized. One donor ovary was implanted in the recipient's ovarian bursa. After peritoneal and skin suture, recipients were allowed to recover for 2 weeks. Ovaries were then analyzed at that stage or recipient mice were mated with WT males to evaluate fertility. For mammary gland transplantation, 12 week-old WT and KO donor mice were used to obtain mammary glands. Small pieces of donor glands were implanted in the cleared mammary fat pad of one gland of 2.7 week old WT mice under general anesthesia. The contra-lateral mammary gland was cleared and kept as a control for clearing. Recipient mice were sacrificed at 12 weeks and the transplanted mammary glands were analyzed by whole mount.

Statistics

Statistical analyses were carried out using unpaired Mann-Whitney test or when indicated Fisher exact test or Kruskal-Wallis test followed by Dunn's multiple comparison. To assess biological interpretation of the most differentially expressed genes, Gene ontology (GO) enrichment analysis was performed using the DAVID bioinformatics resources (<https://david.ncifcrf.gov>, Version 6.8).

DATA AVAILABILITY

The datasets discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al*, 2002) and are accessible through GEO Series accession number GSE144231 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE144231>).

COMPETING OF INTERESTS

The authors declare that they have no conflict of interest to disclose.

AUTHORS CONTRIBUTIONS

CT, IRL, CB, LG, LLC, KC, AG performed experiments. KC, PM, GL and KB contributed to the design of the experiments and to the editing of the manuscript. GL supervised the project, designed the experiments, performed experiments, analyzed the data and wrote the paper.

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REFERENCES

1. Pritchett-Corning KR, Cosentino J, Clifford CB (2009) Contemporary prevalence of infectious agents in laboratory mice and rats. *Laboratory animals* 43:165-173
2. Beura LK, Hamilton SE, Bi K, Schenkel JM, Odumade OA, Casey KA, Thompson EA, Fraser KA, Rosato PC, Filali-Mouhim A, et al. (2016) Normalizing the environment recapitulates adult human immune traits in laboratory mice. *Nature* 532:512-516
3. Reese TA, Bi K, Kambal A, Filali-Mouhim A, Beura LK, Burger MC, Pulendran B, Sekaly RP, Jameson SC, Masopust D, et al. (2016) Sequential Infection with Common Pathogens Promotes Human-like Immune Gene Expression and Altered Vaccine Response. *Cell host & microbe* 19:713-719
4. Rosshart SP, Herz J, Vassallo BG, Hunter A, Wall MK, Badger JH, McCulloch JA, Anastasakis DG, Sarshad AA, Leonardi I, et al. (2019) Laboratory mice born to wild mice have natural microbiota and model human immune responses. *Science* 365
5. Cypowyj S, Picard C, Marodi L, Casanova JL, Puel A (2012) Immunity to infection in IL-17-deficient mice and humans. *Eur J Immunol* 42:2246-2254
6. McCafferty J, Muhlbauer M, Gharaibeh RZ, Arthur JC, Perez-Chanona E, Sha W, Jobin C, Fodor AA (2013) Stochastic changes over time and not founder effects drive cage effects in microbial community assembly in a mouse model. *The ISME journal* 7:2116-2125
7. Hooper LV, Littman DR, Macpherson AJ (2012) Interactions between the microbiota and the immune system. *Science* 336:1268-1273
8. Honda K, Littman DR (2016) The microbiota in adaptive immune homeostasis and disease. *Nature* 535:75-84
9. Bloom SM, Bijanki VN, Nava GM, Sun L, Malvin NP, Donermeyer DL, Dunne WM, Jr., Allen PM, Stappenbeck TS (2011) Commensal *Bacteroides* species induce colitis in host-genotype-specific fashion in a mouse model of inflammatory bowel disease. *Cell host & microbe* 9:390-403
10. Cadwell K, Patel KK, Maloney NS, Liu TC, Ng AC, Storer CE, Head RD, Xavier R, Stappenbeck TS, Virgin HW (2010) Virus-plus-susceptibility gene interaction determines Crohn's disease gene *Atg16L1* phenotypes in intestine. *Cell* 141:1135-1145
11. Wang Z, Roberts AB, Buffa JA, Levison BS, Zhu W, Org E, Gu X, Huang Y, Zamanian-Daryoush M, Culley MK, et al. (2015) Non-lethal Inhibition of Gut Microbial Trimethylamine Production for the Treatment of Atherosclerosis. *Cell* 163:1585-1595
12. Scher JU, Sczesnak A, Longman RS, Segata N, Ubeda C, Bielski C, Rostron T, Cerundolo V, Pamer EG, Abramson SB, et al. (2013) Expansion of intestinal *Prevotella copri* correlates with enhanced susceptibility to arthritis. *eLife* 2:e01202
13. Thorburn AN, McKenzie CI, Shen S, Stanley D, Macia L, Mason LJ, Roberts LK, Wong CH, Shim R, Robert R, et al. (2015) Evidence that asthma is a developmental origin disease influenced by maternal diet and bacterial metabolites. *Nat Commun* 6:7320
14. Roy S, Trinchieri G (2017) Microbiota: a key orchestrator of cancer therapy. *Nat Rev Cancer* 17:271-285
15. Stappenbeck TS, Virgin HW (2016) Accounting for reciprocal host-microbiome interactions in experimental science. *Nature* 534:191-199
16. Cadwell K, Liu JY, Brown SL, Miyoshi H, Loh J, Lennerz JK, Kishi C, Kc W, Carrero JA, Hunt S, et al. (2008) A key role for autophagy and the autophagy gene *Atg16l1* in mouse and human intestinal Paneth cells. *Nature* 456:259-263
17. Okada H, Kuhn C, Feillet H, Bach JF (2010) The 'hygiene hypothesis' for autoimmune and allergic diseases: an update. *Clin Exp Immunol* 160:1-9
18. Bach JF (2002) The effect of infections on susceptibility to autoimmune and allergic diseases. *N Engl J Med* 347:911-920
19. Ramanan D, Tang MS, Bowcutt R, Loke P, Cadwell K (2014) Bacterial sensor *Nod2* prevents inflammation of the small intestine by restricting the expansion of the commensal *Bacteroides vulgatus*. *Immunity* 41:311-324
20. Ramanan D, Bowcutt R, Lee SC, Tang MS, Kurtz ZD, Ding Y, Honda K, Gause WC, Blaser MJ, Bonneau RA, et al. (2016) Helminth infection promotes colonization resistance via type 2 immunity. *Science* 352:608-612
21. Tao L, Reese TA (2017) Making Mouse Models That Reflect Human Immune Responses. *Trends Immunol* 38:181-193
22. Zlotnik A, Yoshie O (2000) Chemokines: a new classification system and their role in immunity. *Immunity* 12:121-127
23. Wang J, Knaut H (2014) Chemokine signaling in development and disease. *Development* 141:4199-4205
24. McCully ML, Kouzeli A, Moser B (2018) Peripheral Tissue Chemokines: Homeostatic Control of Immune Surveillance T Cells. *Trends Immunol* 39:734-747

25. Strieter RM, Burdick MD, Gomperts BN, Belperio JA, Keane MP (2005) CXC chemokines in angiogenesis. *Cytokine Growth Factor Rev* 16:593-609
26. Chavey C, Lazennec G, Lagarrigue S, Clape C, Iankova I, Teyssier J, Annicotte JS, Schmidt J, Matakci C, Yamamoto H, et al. (2009) CXC ligand 5 is an adipose-tissue derived factor that links obesity to insulin resistance. *Cell Metab* 9:339-349
27. Henrot P, Prevel R, Berger P, Dupin I (2019) Chemokines in COPD: From Implication to Therapeutic Use. *International journal of molecular sciences* 20
28. Lazennec G, Lam PY (2016) Recent discoveries concerning the tumor - mesenchymal stem cell interactions. *Biochim Biophys Acta* 1866:290-299
29. Lazennec G, Richmond A (2010) Chemokines and chemokine receptors: new insights into cancer-related inflammation. *Trends Mol Med* 16:133-144
30. Devalaraja RM, Nanney LB, Du J, Qian Q, Yu Y, Devalaraja MN, Richmond A (2000) Delayed wound healing in CXCR2 knockout mice. *J Invest Dermatol* 115:234-244
31. Addison CL, Daniel TO, Burdick MD, Liu H, Ehler JE, Xue YY, Buechi L, Walz A, Richmond A, Strieter RM (2000) The CXC chemokine receptor 2, CXCR2, is the putative receptor for ELR+ CXC chemokine-induced angiogenic activity. *J Immunol* 165:5269-5277
32. Liu L, Belkadi A, Darnall L, Hu T, Drescher C, Coteleur AC, Padovani-Claudio D, He T, Choi K, Lane TE, et al. (2010) CXCR2-positive neutrophils are essential for cuprizone-induced demyelination: relevance to multiple sclerosis. *Nature neuroscience* 13:319-326
33. Tsai HH, Frost E, To V, Robinson S, Ffrench-Constant C, Geertman R, Ransohoff RM, Miller RH (2002) The chemokine receptor CXCR2 controls positioning of oligodendrocyte precursors in developing spinal cord by arresting their migration. *Cell* 110:373-383
34. Boisvert WA, Curtiss LK, Terkeltaub RA (2000) Interleukin-8 and its receptor CXCR2 in atherosclerosis. *Immunol Res* 21:129-137
35. Strieter RM, Keane MP, Burdick MD, Sakkour A, Murray LA, Belperio JA (2005) The role of CXCR2/CXCR2 ligands in acute lung injury. *Curr Drug Targets Inflamm Allergy* 4:299-303
36. Cummings CJ, Martin TR, Frevert CW, Quan JM, Wong VA, Mongovin SM, Hagen TR, Steinberg KP, Goodman RB (1999) Expression and function of the chemokine receptors CXCR1 and CXCR2 in sepsis. *J Immunol* 162:2341-2346
37. Ali S, Lazennec G (2007) Chemokines: novel targets for breast cancer metastasis. *Cancer Metastasis Rev* 26:401-420
38. Freund A, Chauveau C, Brouillet JP, Lucas A, Lacroix M, Licznar A, Vignon F, Lazennec G (2003) IL-8 expression and its possible relationship with estrogen-receptor-negative status of breast cancer cells. *Oncogene* 22:256-265
39. Bieche I, Chavey C, Andrieu C, Busson M, Vacher S, Le Corre L, Guinebretiere JM, Burlinchon S, Lidereau R, Lazennec G (2007) CXC chemokines located in the 4q21 region are up-regulated in breast cancer. *Endocr Relat Cancer* 14:1039-1052
40. Cacalano G, Lee J, Kikly K, Ryan AM, Pitts-Meek S, Hultgren B, Wood WI, Moore MW (1994) Neutrophil and B cell expansion in mice that lack the murine IL-8 receptor homolog. *Science* 265:682-684
41. Broxmeyer HE, Cooper S, Cacalano G, Hague NL, Bailish E, Moore MW (1996) Involvement of Interleukin (IL) 8 receptor in negative regulation of myeloid progenitor cells in vivo: evidence from mice lacking the murine IL-8 receptor homolog. *J Exp Med* 184:1825-1832
42. Freund A, Jolivel V, Durand S, Kersual N, Chalbos D, Chavey C, Vignon F, Lazennec G (2004) Mechanisms underlying differential expression of interleukin-8 in breast cancer cells. *Oncogene* 23:6105-6114
43. Kelly PA, Bachelot A, Kedzia C, Hennighausen L, Ormandy CJ, Kopchick JJ, Binart N (2002) The role of prolactin and growth hormone in mammary gland development. *Mol Cell Endocrinol* 197:127-131
44. Luetke NC, Qiu TH, Fenton SE, Troyer KL, Riedel RF, Chang A, Lee DC (1999) Targeted inactivation of the EGF and amphiregulin genes reveals distinct roles for EGF receptor ligands in mouse mammary gland development. *Development* 126:2739-2750
45. Kouros-Mehr H, Slorach EM, Sternlicht MD, Werb Z (2006) GATA-3 maintains the differentiation of the luminal cell fate in the mammary gland. *Cell* 127:1041-1055
46. Zhou J, Chehab R, Tkalcovic J, Naylor MJ, Harris J, Wilson TJ, Tsao S, Tellis I, Zavarsek S, Xu D, et al. (2005) E1f5 is essential for early embryogenesis and mammary gland development during pregnancy and lactation. *EMBO J* 24:635-644
47. Watson CJ, Khaled WT (2008) Mammary development in the embryo and adult: a journey of morphogenesis and commitment. *Development* 135:995-1003
48. Piekorz RP, Gingras S, Hoffmeyer A, Ihle JN, Weinstein Y (2005) Regulation of progesterone levels during pregnancy and parturition by signal transducer and activator of transcription 5 and 20alpha-hydroxysteroid dehydrogenase. *Mol Endocrinol* 19:431-440

49. Rosenfeld CS, Roberts RM, Lubahn DB (2001) Estrogen receptor- and aromatase-deficient mice provide insight into the roles of estrogen within the ovary and uterus. *Molecular reproduction and development* 59:336-346
50. Payne AH, Clarke TR, Bain PA (1995) The murine 3 beta-hydroxysteroid dehydrogenase multigene family: structure, function and tissue-specific expression. *J Steroid Biochem Mol Biol* 53:111-118
51. Stocco C, Telleria C, Gibori G (2007) The molecular control of corpus luteum formation, function, and regression. *Endocr Rev* 28:117-149
52. Bachelot A, Binart N (2005) Corpus luteum development: lessons from genetic models in mice. *Current topics in developmental biology* 68:49-84
53. Shiina H, Matsumoto T, Sato T, Igarashi K, Miyamoto J, Takemasa S, Sakari M, Takada I, Nakamura T, Metzger D, et al. (2006) Premature ovarian failure in androgen receptor-deficient mice. *Proc Natl Acad Sci U S A* 103:224-229
54. Walters KA (2015) Role of androgens in normal and pathological ovarian function. *Reproduction* 149:R193-218
55. Behringer R (2017) Mouse Ovary Transplantation. *Cold Spring Harbor protocols* 2017
56. Choi JH, Ishida M, Matsuwaki T, Yamanouchi K, Nishihara M (2008) Involvement of 20alpha-hydroxysteroid dehydrogenase in the maintenance of pregnancy in mice. *The Journal of reproduction and development* 54:408-412
57. Ishida M, Choi JH, Hirabayashi K, Matsuwaki T, Suzuki M, Yamanouchi K, Horai R, Sudo K, Iwakura Y, Nishihara M (2007) Reproductive phenotypes in mice with targeted disruption of the 20alpha-hydroxysteroid dehydrogenase gene. *The Journal of reproduction and development* 53:499-508
58. Toda K, Takeda K, Okada T, Akira S, Saibara T, Kaname T, Yamamura K, Onishi S, Shizuta Y (2001) Targeted disruption of the aromatase P450 gene (Cyp19) in mice and their ovarian and uterine responses to 17beta-oestradiol. *J Endocrinol* 170:99-111
59. Chapman JC, Polanco JR, Min S, Michael SD (2005) Mitochondrial 3 beta-hydroxysteroid dehydrogenase (HSD) is essential for the synthesis of progesterone by corpora lutea: an hypothesis. *Reproductive biology and endocrinology : RB&E* 3:11
60. Horseman ND, Zhao W, Montecino-Rodriguez E, Tanaka M, Nakashima K, Engle SJ, Smith F, Markoff E, Dorshkind K (1997) Defective mammopoiesis, but normal hematopoiesis, in mice with a targeted disruption of the prolactin gene. *EMBO J* 16:6926-6935
61. Yanaihara A, Mitsukawa K, Iwasaki S, Otsuki K, Kawamura T, Okai T (2007) High concentrations of lactoferrin in the follicular fluid correlate with embryo quality during in vitro fertilization cycles. *Fertil Steril* 87:279-282
62. Hu YC, Wang PH, Yeh S, Wang RS, Xie C, Xu Q, Zhou X, Chao HT, Tsai MY, Chang C (2004) Subfertility and defective folliculogenesis in female mice lacking androgen receptor. *Proc Natl Acad Sci U S A* 101:11209-11214
63. Bole-Feysot C, Goffin V, Edery M, Binart N, Kelly PA (1998) Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. *Endocr Rev* 19:225-268
64. Howlin J, McBryan J, Martin F (2006) Pubertal mammary gland development: insights from mouse models. *J Mammary Gland Biol Neoplasia* 11:283-297
65. Jarde T, Dale T (2012) Wnt signalling in murine postnatal mammary gland development. *Acta physiologica* 204:118-127
66. Ciarloni L, Mallepell S, Briskin C (2007) Amphiregulin is an essential mediator of estrogen receptor alpha function in mammary gland development. *Proc Natl Acad Sci U S A* 104:5455-5460
67. Briskin C, Heineman A, Chavarria T, Elenbaas B, Tan J, Dey SK, McMahan JA, McMahan AP, Weinberg RA (2000) Essential function of Wnt-4 in mammary gland development downstream of progesterone signaling. *Genes Dev* 14:650-654
68. Markus MA, Morris BJ (2009) RBM4: a multifunctional RNA-binding protein. *Int J Biochem Cell Biol* 41:740-743
69. Rexach JE, Clark PM, Mason DE, Neve RL, Peters EC, Hsieh-Wilson LC (2012) Dynamic O-GlcNAc modification regulates CREB-mediated gene expression and memory formation. *Nature chemical biology* 8:253-261
70. Chaves I, van der Horst GT, Schellevis R, Nijman RM, Koerkamp MG, Holstege FC, Smidt MP, Hoekman MF (2014) Insulin-FOXO3 signaling modulates circadian rhythms via regulation of clock transcription. *Curr Biol* 24:1248-1255
71. Ketchesin KD, Stinnett GS, Seasholtz AF (2017) Corticotropin-releasing hormone-binding protein and stress: from invertebrates to humans. *Stress* 20:449-464
72. Loh R, Chau L, Aijaz A, Wu K, Galvez R (2017) Antagonizing the different stages of kappa opioid receptor activation selectively and independently attenuates acquisition and consolidation of associative memories. *Behavioural brain research* 323:1-10

73. Hill JM (2007) Vasoactive intestinal peptide in neurodevelopmental disorders: therapeutic potential. *Curr Pharm Des* 13:1079-1089
74. Nirmal AJ, Regan T, Shih BB, Hume DA, Sims AH, Freeman TC (2018) Immune Cell Gene Signatures for Profiling the Microenvironment of Solid Tumors. *Cancer immunology research* 6:1388-1400
75. Bellastella G, Maiorino MI, Bizzarro A, Giugliano D, Esposito K, Bellastella A, De Bellis A (2016) Revisitation of autoimmune hypophysitis: knowledge and uncertainties on pathophysiological and clinical aspects. *Pituitary* 19:625-642
76. Caturegli P, Newschaffer C, Olivi A, Pomper MG, Burger PC, Rose NR (2005) Autoimmune hypophysitis. *Endocr Rev* 26:599-614
77. Appleton J (2018) The Gut-Brain Axis: Influence of Microbiota on Mood and Mental Health. *Integrative medicine* 17:28-32
78. Watanabe K, Gilchrist CA, Uddin MJ, Burgess SL, Abhyankar MM, Moonah SN, Noor Z, Donowitz JR, Schneider BN, Arju T, et al. (2017) Microbiome-mediated neutrophil recruitment via CXCR2 and protection from amebic colitis. *PLoS pathogens* 13:e1006513
79. Wu D, Hong H, Huang X, Huang L, He Z, Fang Q, Luo Y (2016) CXCR2 is decreased in preeclamptic placentas and promotes human trophoblast invasion through the Akt signaling pathway. *Placenta* 43:17-25
80. Chen H, Zhang Y, Dai L, Song Y, Wang Y, Zhou B, Zhou R (2019) Association between polymorphisms in CXCR2 gene and preeclampsia. *Molecular genetics & genomic medicine* 7:e00578
81. Kang X, Zhang X, Liu Z, Xu H, Wang T, He L, Zhao A (2016) CXCR2-Mediated Granulocytic Myeloid-Derived Suppressor Cells' Functional Characterization and Their Role in Maternal Fetal Interface. *DNA Cell Biol* 35:358-365
82. Li JJ, Ma FX, Wang YW, Chen F, Lu SH, Chi Y, Du WJ, Song BQ, Hu LD, Chen H, et al. (2017) Knockdown of IL-8 Provoked Premature Senescence of Placenta-Derived Mesenchymal Stem Cells. *Stem Cells Dev* 26:912-931
83. Sturn A, Quackenbush J, Trajanoski Z (2002) Genesis: cluster analysis of microarray data. *Bioinformatics* 18:207-208
84. Edgar R, Domrachev M, Lash AE (2002) Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res* 30:207-210

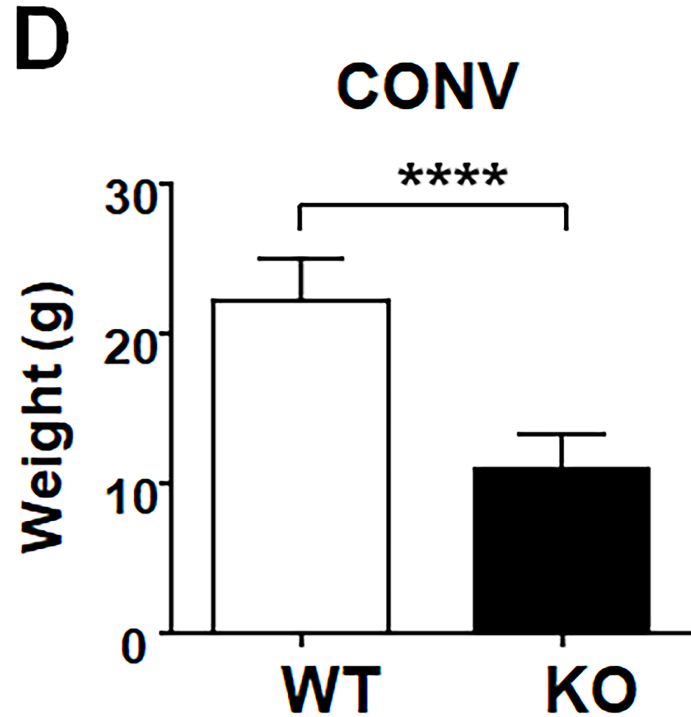
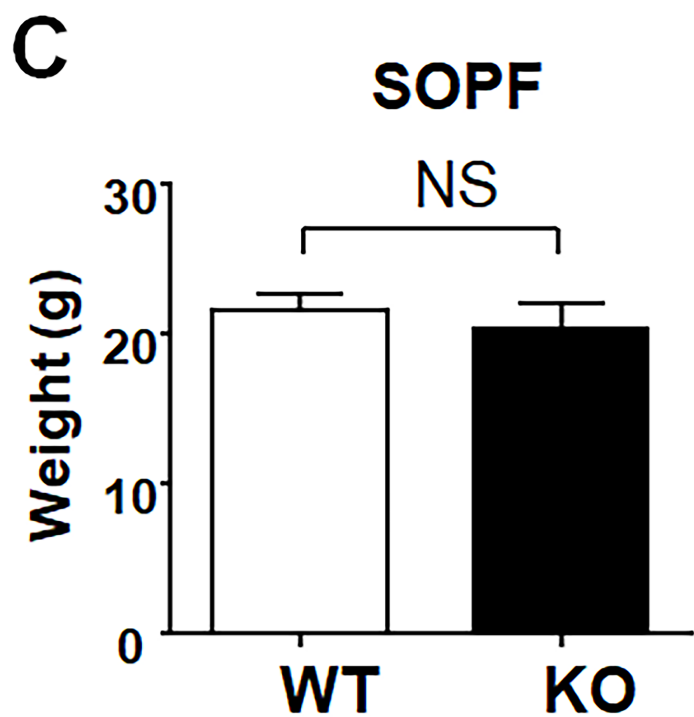
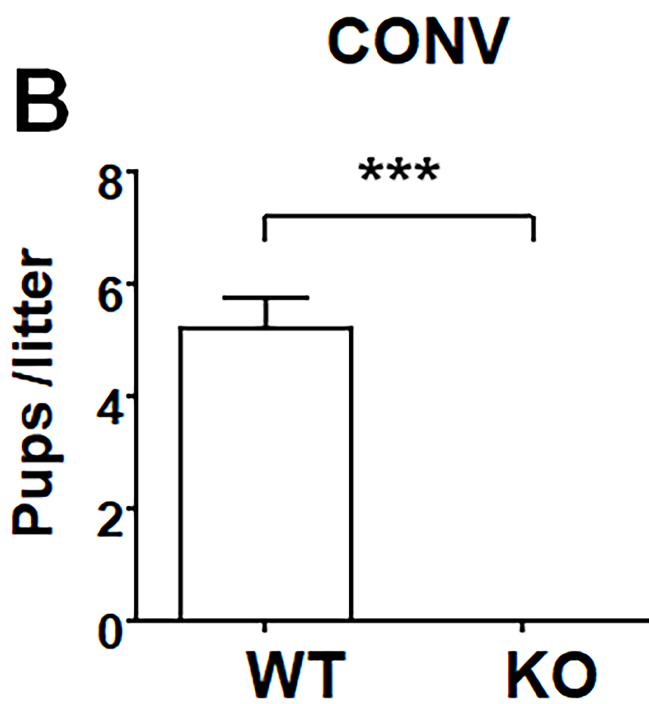
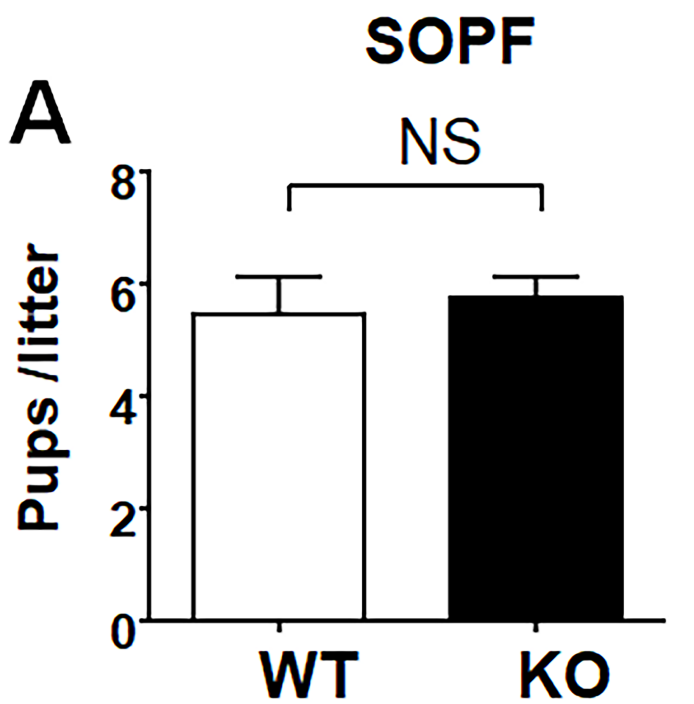


Fig. 1

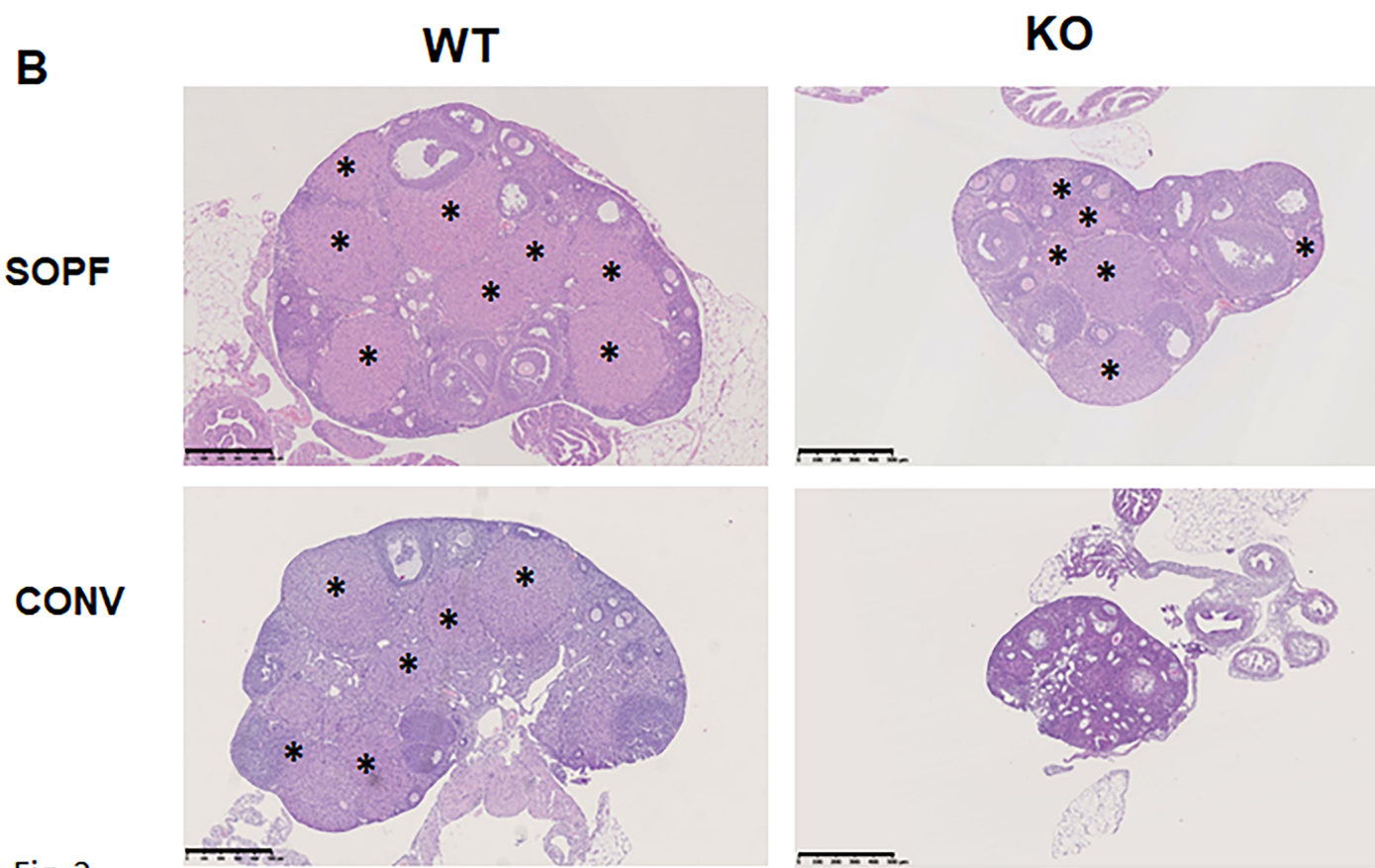
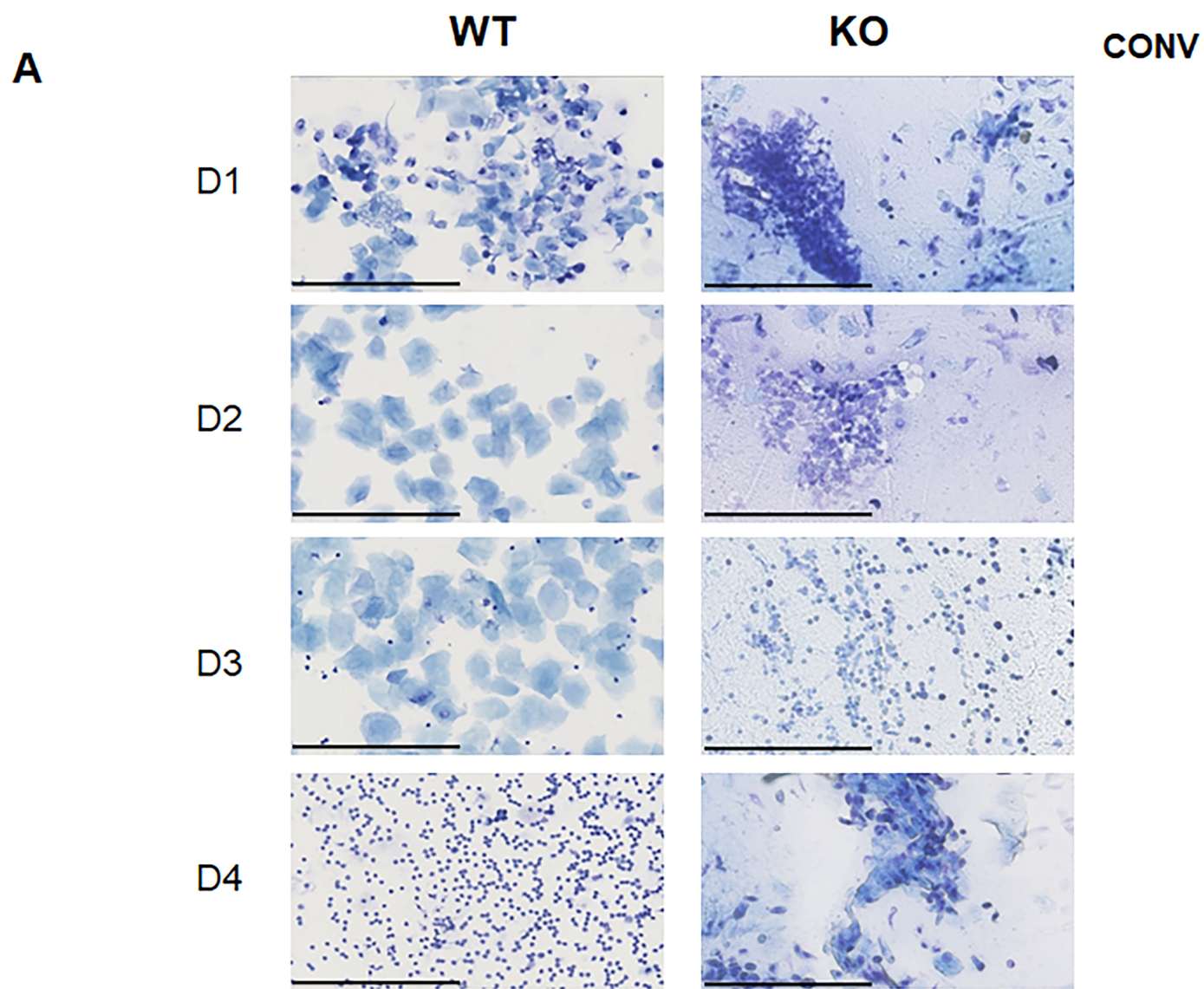


Fig. 2

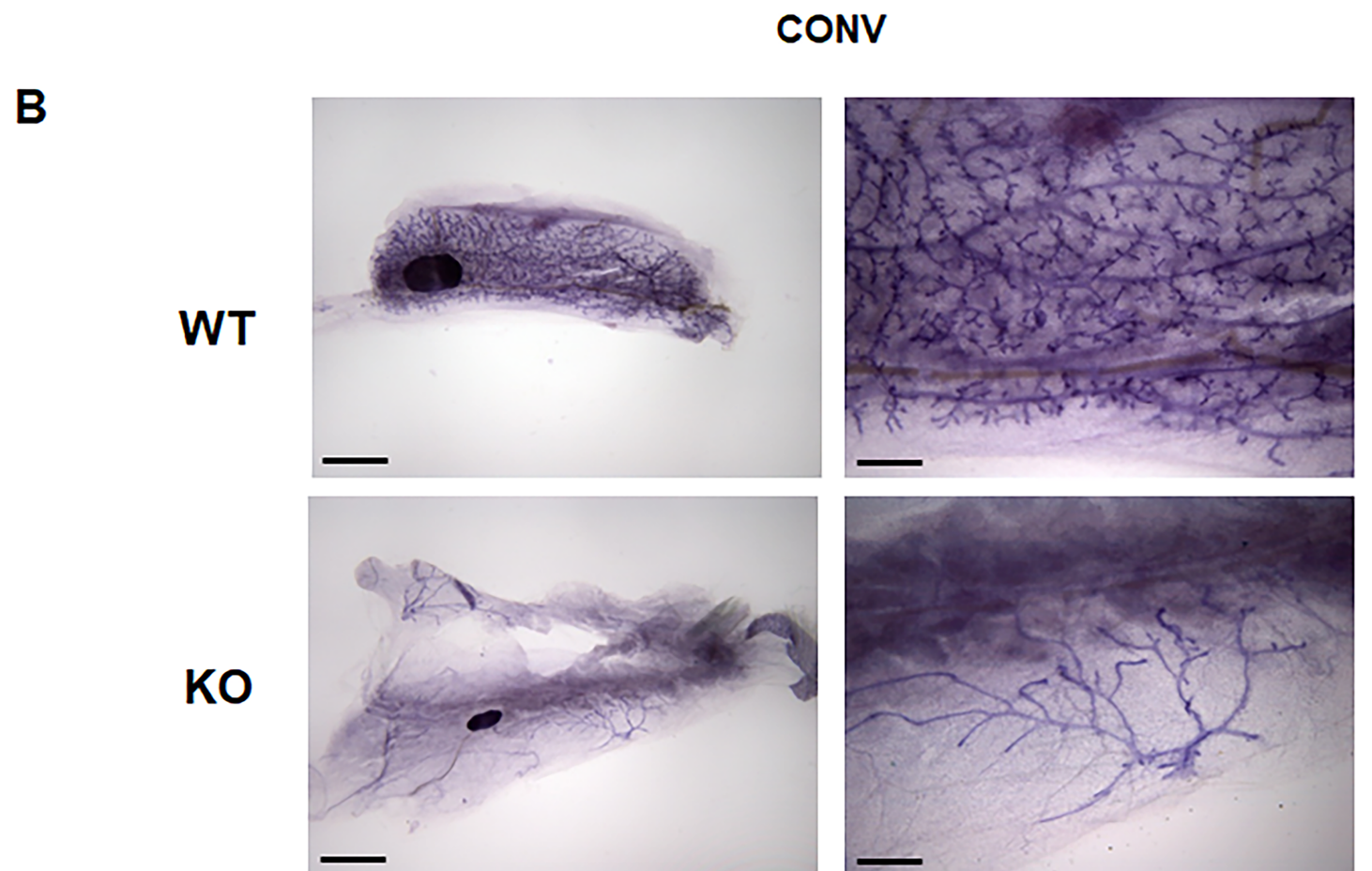
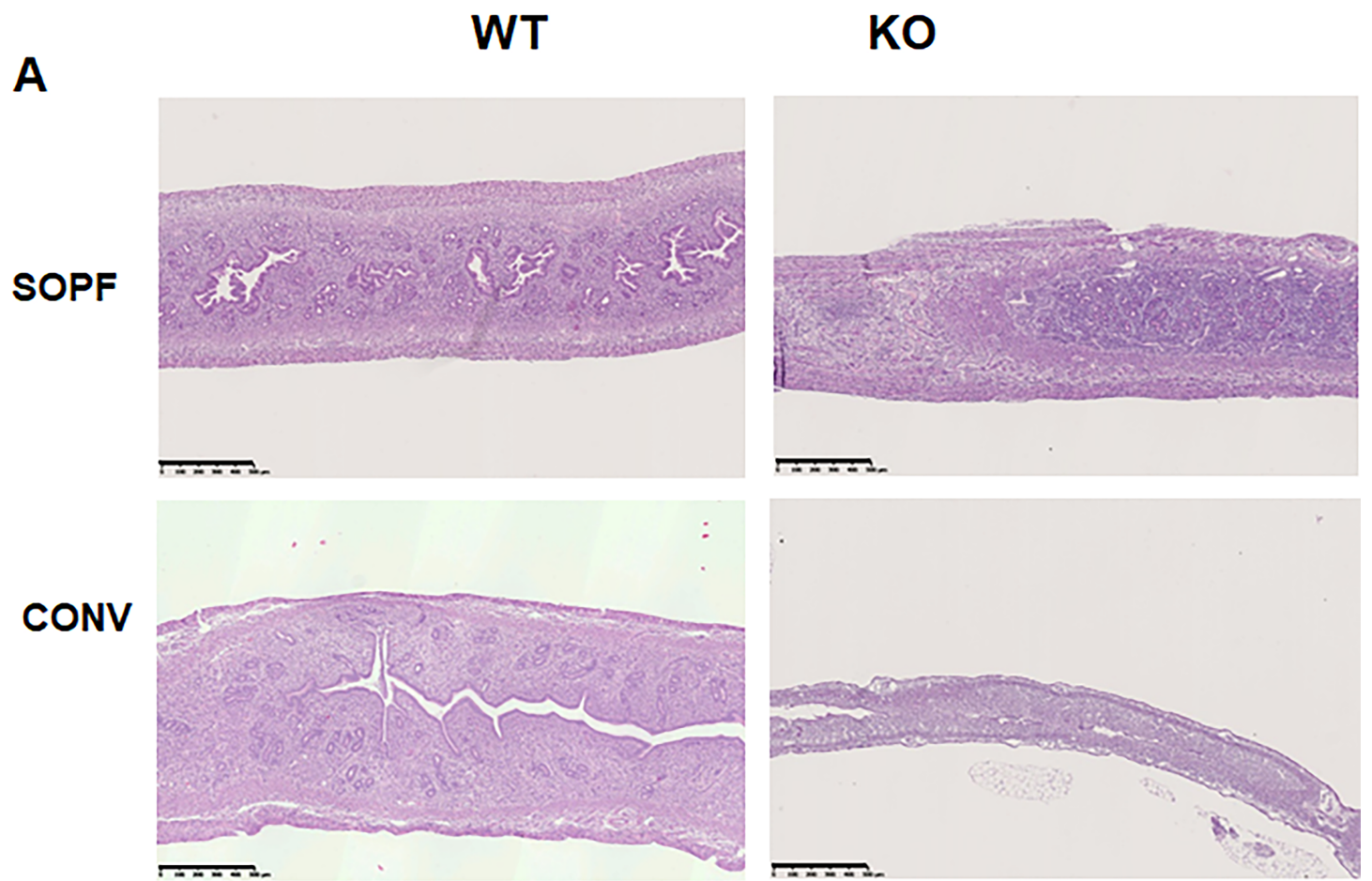
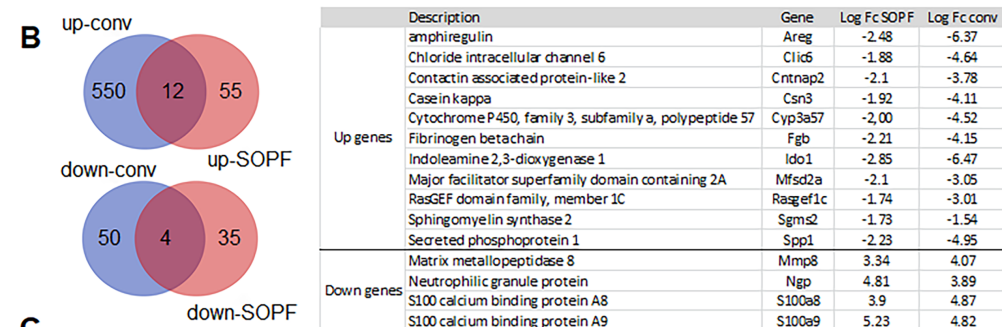
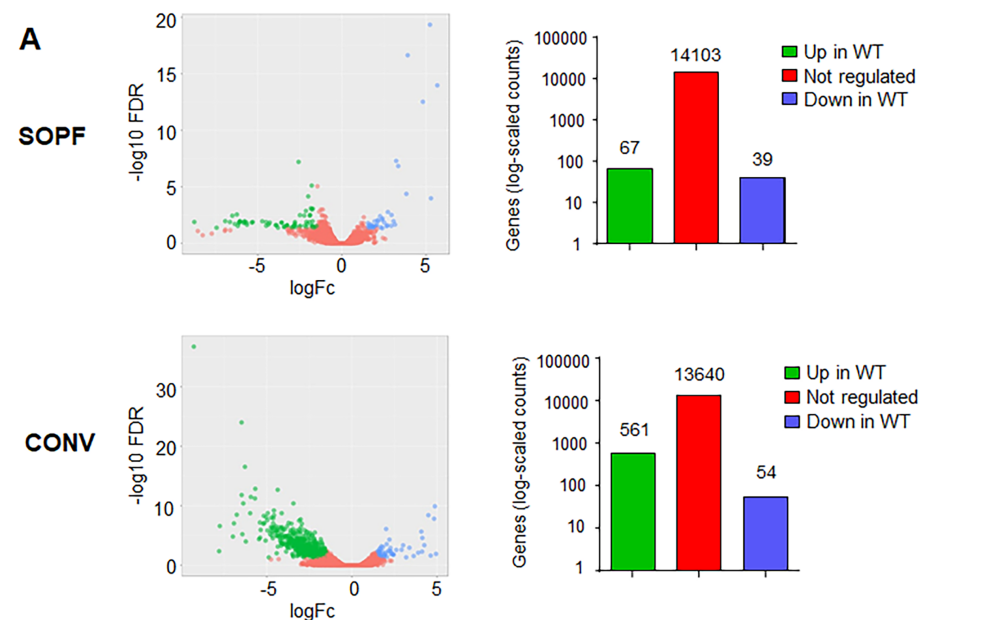


Fig. 3



| BiologicProcess Term | Count | % | PValue | Genes |
|--|-------|-------|---------|---|
| GO:0030593~neutrophil chemotaxis | 4 | 25.0 | 4.69E-5 | Spp1, S100a9, S100a8, Cxcr2 |
| GO:1990266~neutrophil migration | 4 | 25.0 | 7.33E-5 | Spp1, S100a9, S100a8, Cxcr2 |
| GO:0071621~granulocyte chemotaxis | 4 | 25.0 | 8.46E-5 | Spp1, S100a9, S100a8, Cxcr2 |
| GO:0097530~granulocyte migration | 4 | 25.0 | 1.19E-4 | Spp1, S100a9, S100a8, Cxcr2 |
| GO:0032101~regulation of response to external stimulus | 6 | 37.5 | 2.88E-4 | Spp1, S100a9, Ido1, Fgb, S100a8, Cxcr2 |
| GO:0097529~myeloid leukocyte migration | 4 | 25.0 | 3.02E-4 | Spp1, S100a9, S100a8, Cxcr2 |
| GO:0030595~leukocyte chemotaxis | 4 | 25.0 | 5.18E-4 | Spp1, S100a9, S100a8, Cxcr2 |
| GO:0006952~defense response | 7 | 43.75 | 7.82E-4 | Spp1, S100a9, Ido1, Fgb, Ngp, Cxcr2, S100a8 |

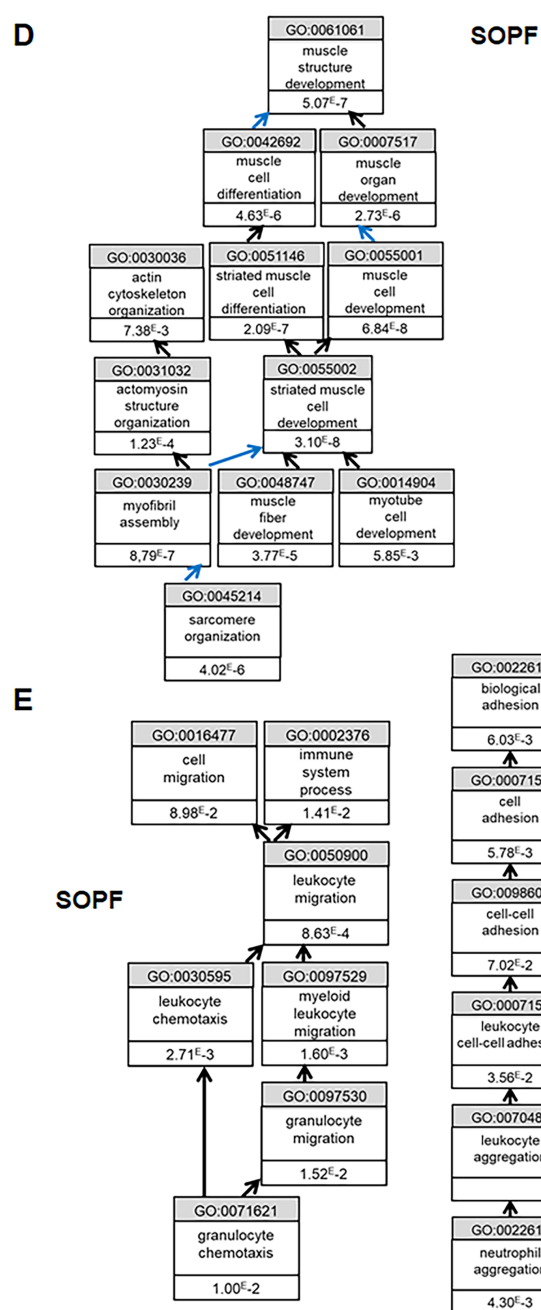


Fig. 4

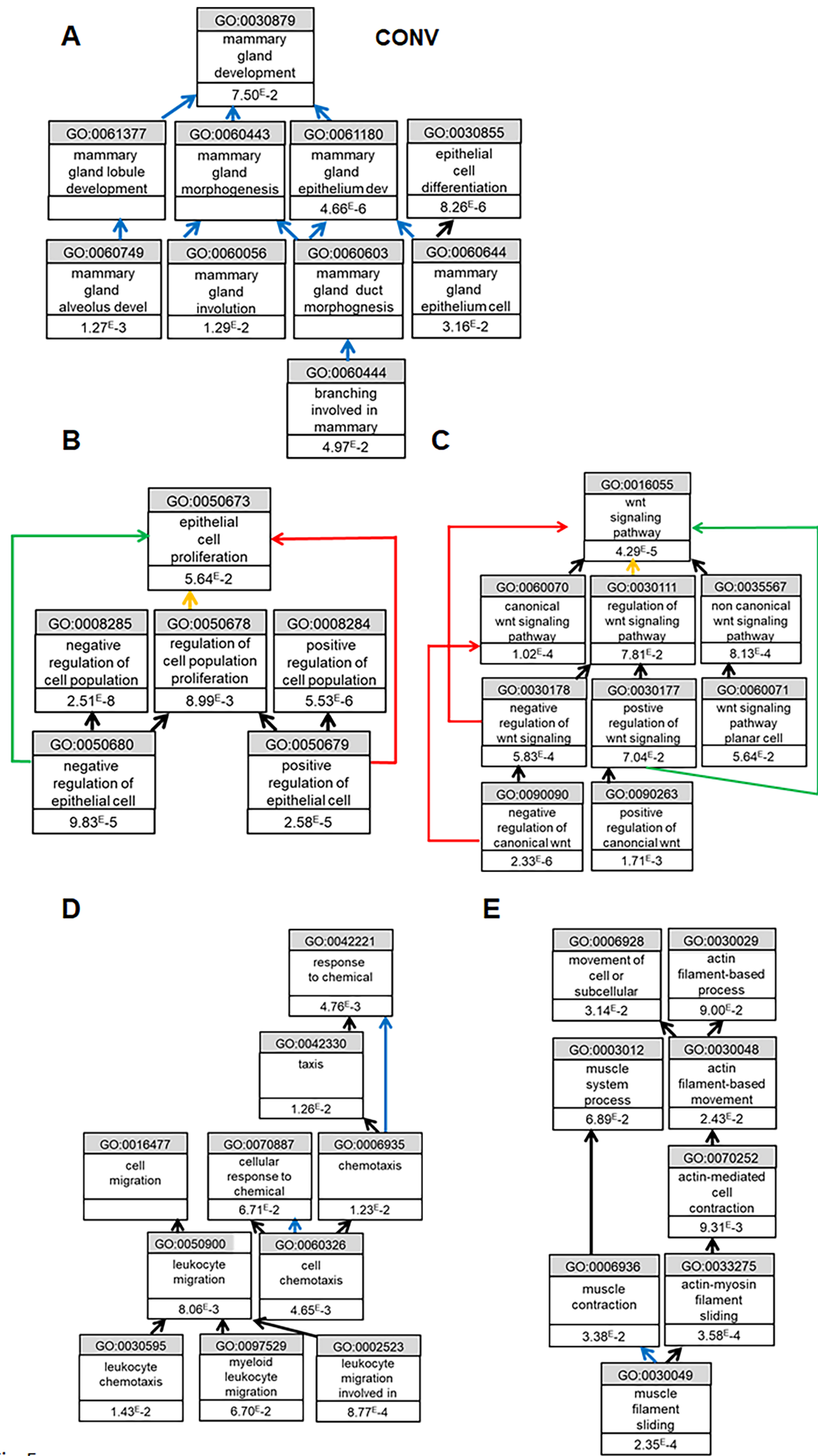


Fig. 5

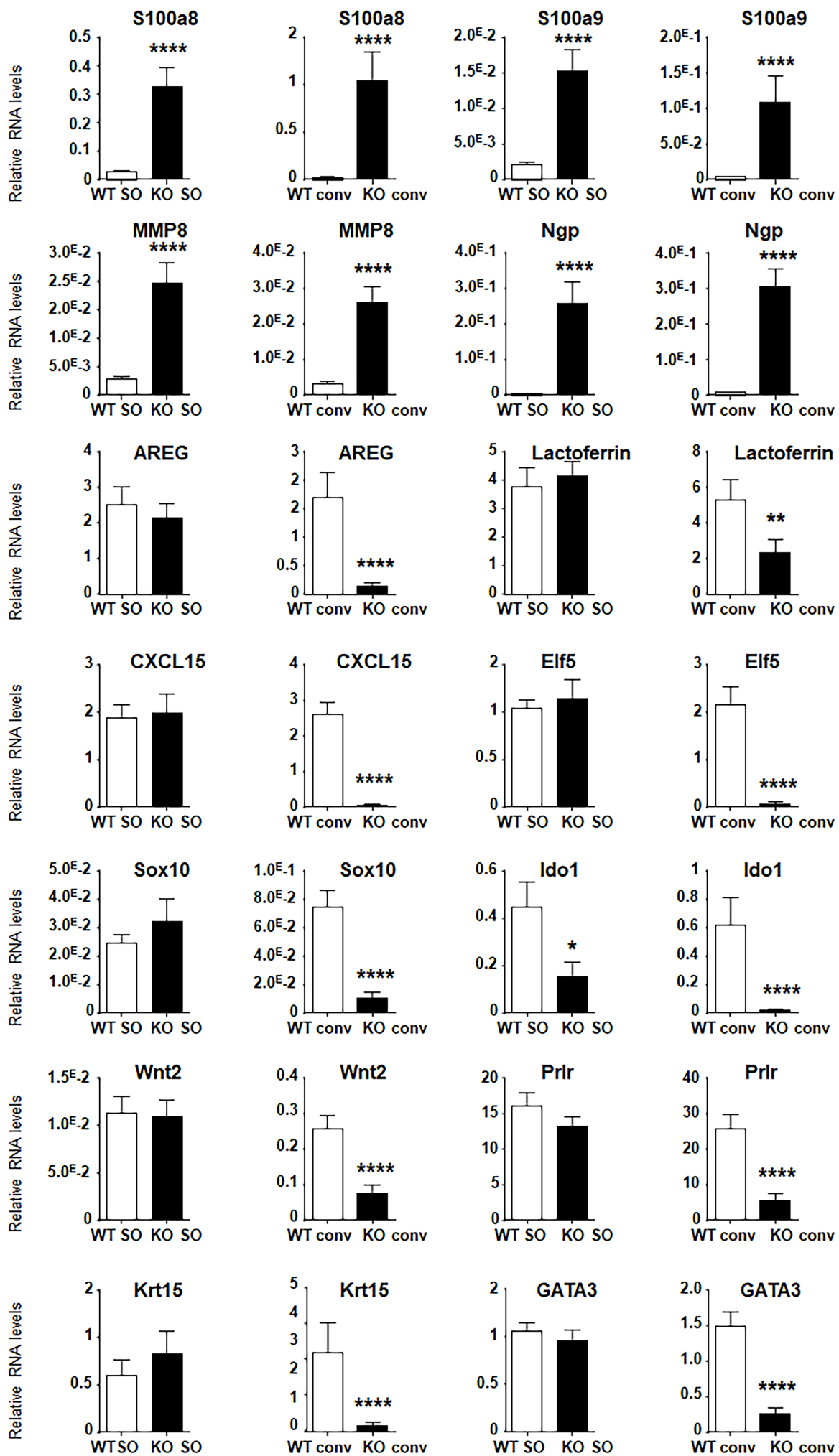
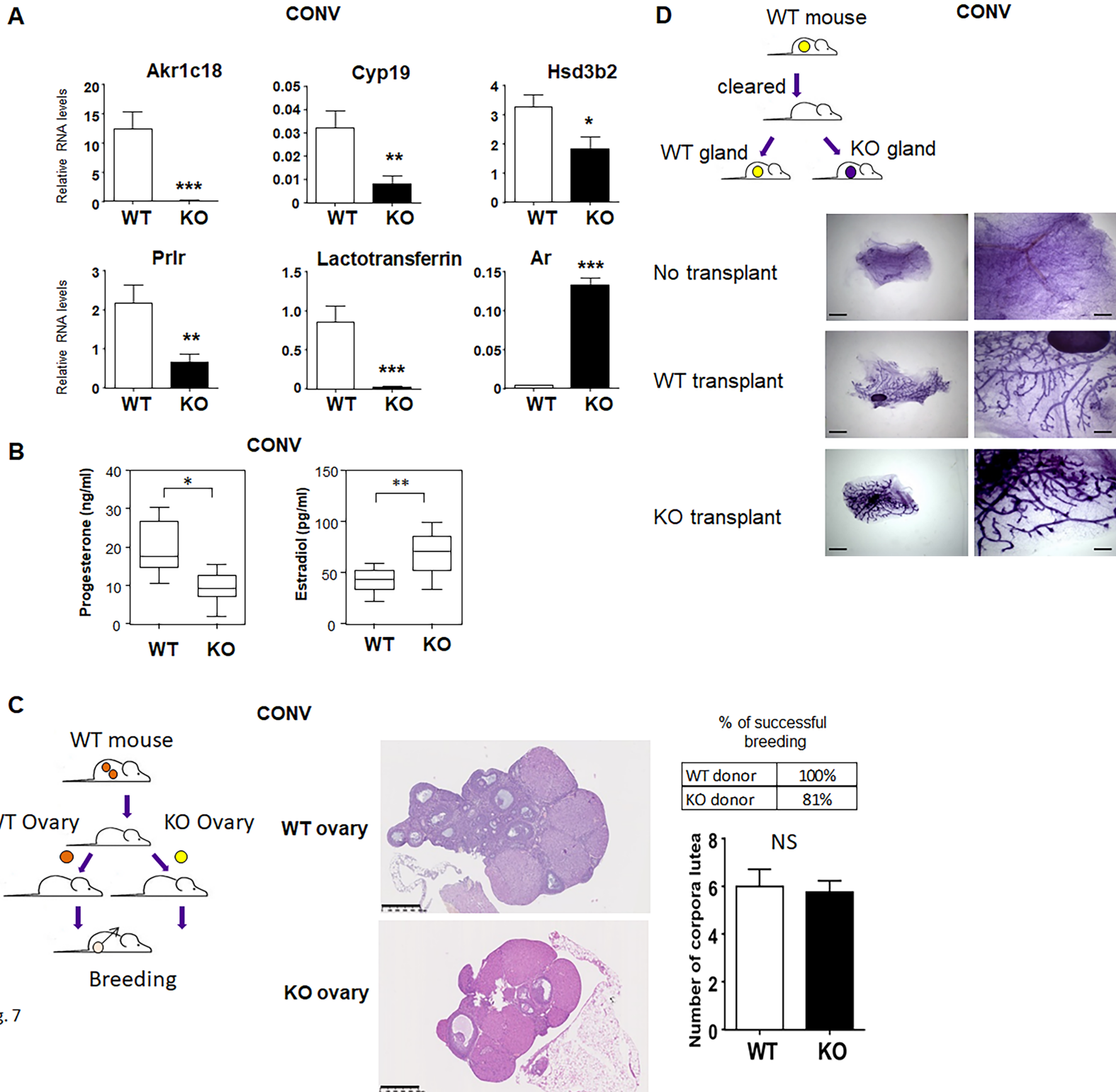


Fig. 6



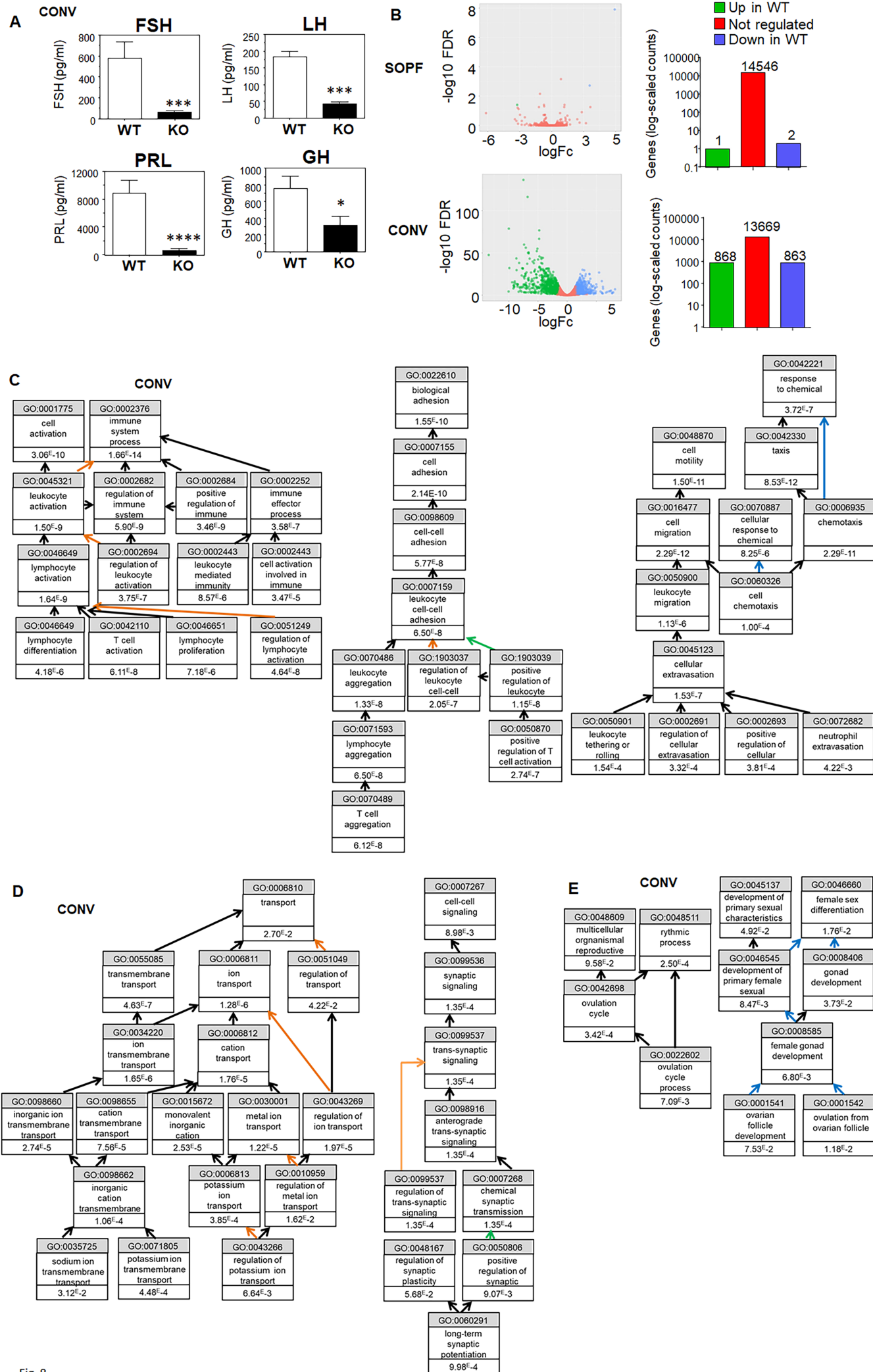


Fig. 8

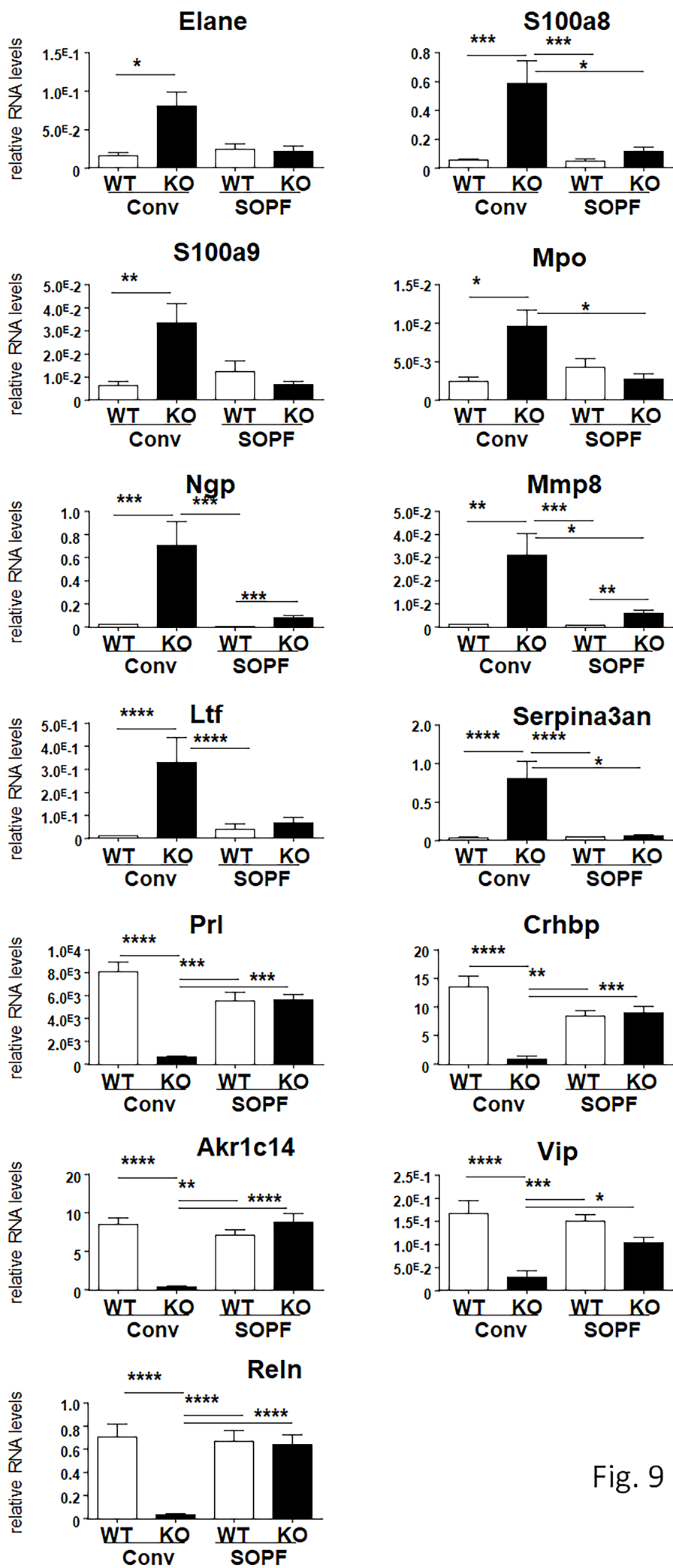


Fig. 9

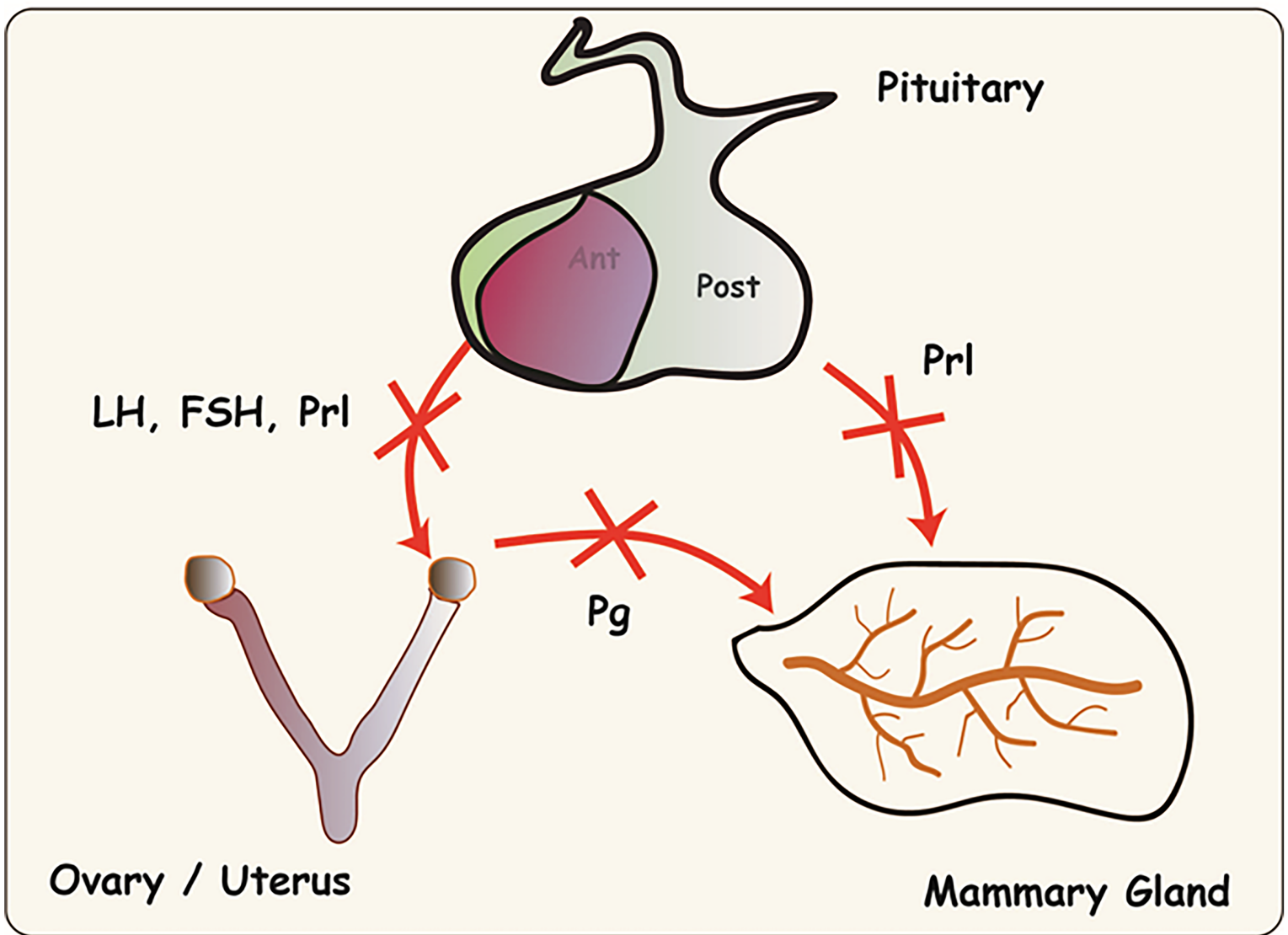
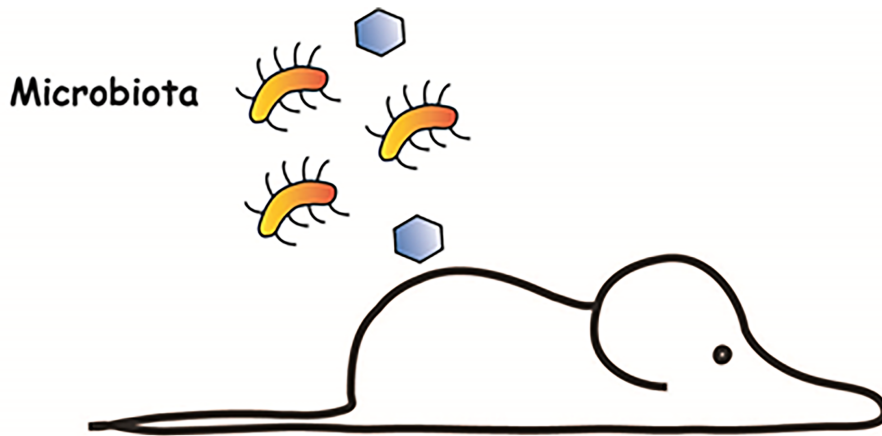


Fig. 10