








Cell adhesion and immune response, two main functions altered in the transcriptome of seasonally regressed testes of two mammalian species

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Abstract

In species with seasonal breeding, male specimens undergo substantial testicular regression during the nonbreeding period of the year. However, the molecular mechanisms that control this biological process are largely unknown. Here, we report a transcriptomic analysis on the Iberian mole, *Talpa occidentalis*, in which the desquamation of live, nonapoptotic germ cells is the major cellular event responsible for testis regression. By comparing testes at different reproductive states (active, regressing, and inactive), we demonstrate that the molecular pathways controlling the cell adhesion function in the seminiferous epithelium, such as the MAPK, ERK, and TGF- β signaling, are altered during the regression process. In addition, inactive testes display a global upregulation of genes associated with immune response, indicating a selective loss of the “immune privilege” that normally operates in sexually active testes. Interspecies comparative analyses using analogous data from the Mediterranean pine vole, a rodent species where testis regression is controlled by halting meiosis entry, revealed a common gene expression signature in the regressed testes of these two evolutionary distant species. Our study advances in the knowledge of the molecular mechanisms associated to gonadal seasonal breeding, highlighting the existence of a conserved transcriptional program of testis involution across mammalian clades.

KEYWORDS

cell adhesion, immune response, *Microtus duodecimcostatus*, seasonal reproduction, seasonal testis regression, *Talpa occidentalis*, testis transcriptome

Francisca M. Real and Miguel Lao-Pérez should be considered joint first author. Rafael Jiménez and Francisco J. Barrionuevo should be considered joint senior author.

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1 | INTRODUCTION

In temperate zones of the Earth, most species reproduce during the season that offers the best conditions for breeding success. In the transition period between the reproductive and the nonreproductive seasons, the gonads of both sexes undergo substantial changes, whose nature is species-specific (Jiménez et al., 2015). In females, ovaries entry in anoestrus (Das & Khan, 2010) and sexual receptivity is either reduced or abolished, as shown in the musk shrew, *Suncus murinus* (Temple, 2004). In males of several species, a process of testis regression takes place by which gonad volume is remarkably reduced and spermatogenesis is arrested, as described in the Syrian hamster, *Mesocricetus auratus* (Martínez-Hernández et al., 2020; Seco-Rovira et al., 2015), the black bear, *Ursus americanus* (Tsubota et al., 1997), the Iberian mole, *Talpa occidentalis* (Dadhich et al., 2010, 2013), the large hairy armadillo *Chaetophractus villosus* (Luaces et al., 2013), the wood mouse, *Apodemus sylvaticus* (Massoud et al., 2021), and the Mediterranean pine vole, *Microtus duodecimcostatus* (Lao-Pérez et al., 2021), among others.

Seasonal breeding relies on circannual modulations of the main regulator of the reproductive system, the hypothalamic–pituitary–gonadal (HPG) axis. In sexually active males the gonadotropin-releasing hormone, GnRH, which is secreted by the hypothalamus, induces the hypophysis (pituitary) to produce and secrete gonadotropic hormones (luteinizing hormone, LH, and follicle-stimulating hormone, FSH) which, in turn, activates both the production of steroids by Leydig cells and the spermatogenic cycle. Environmental cues modulate the function of this axis, being the photoperiod by far the best known, although other factors, such as food and water availability, stress, and weather, can either modify or even overcome the influence of photoperiod (Bronson & Heideman, 1994; Martin et al., 1994; Nelson et al., 1995). In the nonreproductive season, these environmental cues alter the levels of HPG axis hormones, resulting in reduced levels of serum gonadotropins and circulating testosterone, leading to alterations of the spermatogenic cycle and, most frequently, to a halt in gamete production (Dardente et al., 2016).

For many years, germ cell apoptosis was considered to be the only cellular process responsible for germ cell depletion during seasonal testis regression (Pastor et al., 2011; Young & Nelson, 2001). However, more recently, alternative mechanisms have been reported, including germ cell desquamation (sloughing, detachment) (Dadhich et al., 2013; Luaces et al., 2014; Massoud et al., 2018), a combination of apoptosis and autophagy (González et al., 2018), and germ cell de-differentiation (Liu et al., 2016). Despite this, the genetic control of these testicular changes is poorly understood and insufficiently investigated. Expression profiling studies provide both an integrated view of the interacting molecular pathways operating in the testis and relevant information about which of them are altered during seasonal testis regression. The transcription profile of active and inactive testes of seasonal breeding males have been studied in some few mammalian species using either microarray, as in the

Syrian hamster, *M. auratus* (Maywood et al., 2009) or RNA-seq technology, as in the European beaver, *Castor fiber* (Bogacka et al., 2017), the plateau pika, *Ochotona curzoniae* (X. Wang, Adegoke, et al., 2019; Y.-J. Wang, Jia, et al., 2019), and the Mediterranean pine vole, *M. duodecimcostatus* (Lao-Pérez et al., 2021). However, the number and identity of the deregulated genes vary substantially from study to study, a fact that might reflect gene expression differences among species, but it is also likely that these differences are due to the use of distinct profiling technologies and/or bioinformatic analysis. For instance, no data normalization was done in these studies to prevent over-representation of meiotic and postmeiotic germ-cell specific transcripts in the testes of active males (these cell types are absent in the inactive testes). In fact, these disproportionate gene expression differences between active and inactive testes are derived from their different cell contents and do not reflect regulatory changes related to seasonal testis regression. Hence, data must be normalized as these large differences would mask functionally relevant changes in gene expression produced in somatic cells, mainly Sertoli cells (SCs), which do actually regulate the spermatogenic cycle. More species have to be investigated to identify evolutionarily conserved transcriptomic alterations related to seasonal testis regression.

The Iberian mole, *T. occidentalis*, develop sexual features that are unique among mammals, as females consistently develop bilateral ovotestes (gonads with both ovarian and testicular tissue) instead of normal ovaries (Barrionuevo et al., 2004; Jimenez et al., 1993). In addition, moles are strict seasonal breeders. In southern Iberian Peninsula, reproduction occurs during the autumn-winter period (October–March) whereas spring-summer (April–September) is the quiescence season. In summer, circulating testosterone levels are reduced and the regressed (inactive) testis shrinks to one-fourth of their winter volume and mass. This testis regression is mediated by desquamation of live, nonapoptotic germ cells occurring in spring (April–May). In the regressed testes, spermatogonia continue entering meiosis, but spermatogenesis does not progress beyond the primary spermatocyte stage (pachytene), as meiotic cells are eliminated by apoptosis. Also, the expression and distribution of the cell-adhesion molecules in the seminiferous epithelium are altered, and the blood-testis barrier (BTB) becomes permeable (Dadhich et al., 2010, 2011, 2013).

We have recently sequenced and annotated the genome of *T. occidentalis*, shedding light on the genomic changes and molecular adaptations that lead to female ovotestis formation (Real et al., 2020). Using this resource, we have now explored the genetic control of the changes that the testis of the Iberian mole undergoes during the process of testicular regression. By performing a transcriptomic analysis of active, regressing, and inactive testes, we demonstrate that pathways such as extracellular matrix organization and cell junction assembly are derregulated during testis regression, as well as the molecular pathways that control these processes during normal testicular function, mainly the MAPK signaling pathway. We also found that inactive testes have lost the “immune privilege” (reduced immune response) that operates normally in active testes. Finally, we performed an inter-species comparative analysis against analogous

datasets we reported for the Mediterranean pine vole (Lao-Pérez et al., 2021), finding that a large number of genes are commonly deregulated in the inactive testes of both species. These genes are enriched in pathways such as the MAPK and regulation of the immune response, indicating the existence of common molecular mechanisms operating in the regressed testes of seasonal breeding mammals.

2 | RESULTS

2.1 | The expression of genes controlling cell adhesion and immune response is altered in the regressed testes of *T. occidentalis*

As we reported previously (Dadhich et al., 2010, 2013), the testes of Iberian mole males captured in the breeding period (autumn and winter) were four times larger than those of males captured in the nonbreeding period (spring and summer; Figure 1a). These inactive testes contained seminiferous tubules very reduced in diameter and lacked a well-developed germinative epithelium, as spermatogenesis was arrested at the primary spermatocyte stage (Supporting Information: Figure S1). To find differences in gene expression, we performed RNA-seq on active and inactive testes of *T. occidentalis*. Multidimensional scaling plot showed that replicate samples of the same breeding season clustered together, indicating consistent differences in the testis transcriptome between the breeding and the nonbreeding periods (Supporting Information: Figure S2). Before normalization for differences in germ cell contents between active and inactive testes, differential expression analysis of RNA-seq data revealed 7049 differentially expressed genes (DEGs) between the two breeding periods, from which 3365 were upregulated (up-DEGs) and 3684 downregulated (down-DEGs) in the regressed testes ($FDR < 0.05$ and $|\log_2FC| > 1$; Supporting Information: Figure S3; Supporting Information: Table S1). GO analysis of these DEGs showed a significant enrichment ($Padjust < 0.05$) in a number of categories, many of them associated to biological processes occurring during spermatogenesis and spermiogenesis, including “cilium organization” (GO:0044782; $Padjust = 1.9 \times 10^{-6}$), “microtubule-based movement” (GO:0007018; $Padjust = 8 \times 10^{-4}$), spermatogenesis (GO:0007283; $Padjust = 1.7 \times 10^{-3}$), and “sperm motility” (GO:0097722; $Padjust = 6.8 \times 10^{-3}$) among others (Supporting Information: Figure S3; Supporting Information: Table S2). These results evidence the need for a normalization of the data, as most of these GO terms are related to cell contents differences between active and inactive testes, rather than to actual gene expression alterations during testis regression. After normalization, the distance between active and inactive samples was reduced in the multidimensional scaling plot (Supporting Information: Figure S2), indicating that our approach removed in fact differences derived from the distinct germ cell contents between active and inactive testes. In the normalized set of genes, we identified 4327 DEGs, from which 2055 were up-DEGs

and 2272 were down-DEGs (Figure 1b; Supporting Information: Table S3). GO analysis of DEGs and down-DEGs showed very few significant categories ($Padjust < 0.05$; Supporting Information: Tables S4 and S5). In contrast, for the up-DEGs, we found terms related to the cell adhesion function of the seminiferous epithelium, including “regulation of cell adhesion” (GO:0030155; $Padjust = 2 \times 10^{-4}$), “extracellular matrix organization” (GO:0030198; $Padjust = 3 \times 10^{-4}$), “cell junction assembly” (GO:0034329; $Padjust = 2.9 \times 10^{-3}$), and “cell-matrix adhesion” (GO:0007160; $Padjust = 3 \times 10^{-2}$), among others (Figure 1c; Supporting Information: Table S6). We next searched for enriched GO terms related to molecular pathways and we found several GO terms related to SC signaling involved in the regulation of spermatogenesis and BTB dynamics, including “MAPK cascade” (GO:0000165; $Padjust = 4 \times 10^{-3}$) (Ni et al., 2019), “positive regulation of small GTPase mediated signal transduction” (GO:0051057; $Padjust = 3 \times 10^{-2}$; Lui et al., 2003a), “ERK1 and ERK2 cascade” (GO:0070371, $Padjust = 2 \times 10^{-3}$; Zhang et al., 2014), “regulation of cytosolic calcium ion concentration” (GO:0051480; $Padjust = 2 \times 10^{-2}$; Gorczynska & Handelsman, 1995), “response to transforming growth factor beta” (GO:0071559; $Padjust = 9 \times 10^{-3}$; Ni et al., 2019), “Notch signaling pathway” (GO:0007219; $Padjust = 1 \times 10^{-3}$; Garcia et al., 2013), and “canonical Wnt signaling pathway” (GO:0060070; $Padjust = 4 \times 10^{-2}$; X. Wang, Adegoke, et al., 2019; Y.-J. Wang, Jia, et al., 2019) (Figure 1c; Supporting Information: - Table S6). Gene-concept analysis using these data resulted in a large network in which MAPK/ERK1/2 signaling occupied a central position sharing many genes with the other molecular pathways and with the biological process “cell-cell adhesion” (Figure 1d).

The GO analysis of up-DEGs also revealed an enrichment of genes participating in the immune response (Figure 1c; Supporting Information: Table S6) including “positive regulation of NF-kappaB transcription factor activity” (GO:0051092; $Padjust = 2 \times 10^{-3}$), “macrophage activation” (GO:0042116; $Padjust = 1 \times 10^{-2}$), “response to tumor necrosis factor” (GO:0034612; $Padjust = 4 \times 10^{-2}$), “positive regulation of leukocyte activation” (GO:0002696; $Padjust = 3.9 \times 10^{-2}$), “regulation of inflammatory response” (GO:0050727; 1.6×10^{-3}), and “response to cytokine” (GO:0034097; $Padjust = 3 \times 10^{-8}$). Gene-concept analysis using these data generated a network in which both TNF and NF-Kappa signaling share many genes with biological processes involved in the activation of the immune system (Figure 1e).

2.2 | Transcriptome alterations at the onset of testis regression in *T. occidentalis*

Our previous analysis revealed that several molecular pathways are altered in inactive testes when compared to the active ones. However, as these stages represent end-points of the activation-regression cycle, the results might not be indicative of the biological processes that are causative of testis regression. In the population we investigated, males of the Iberian mole undergo testis regression during the months of March and April, when seminiferous tubules

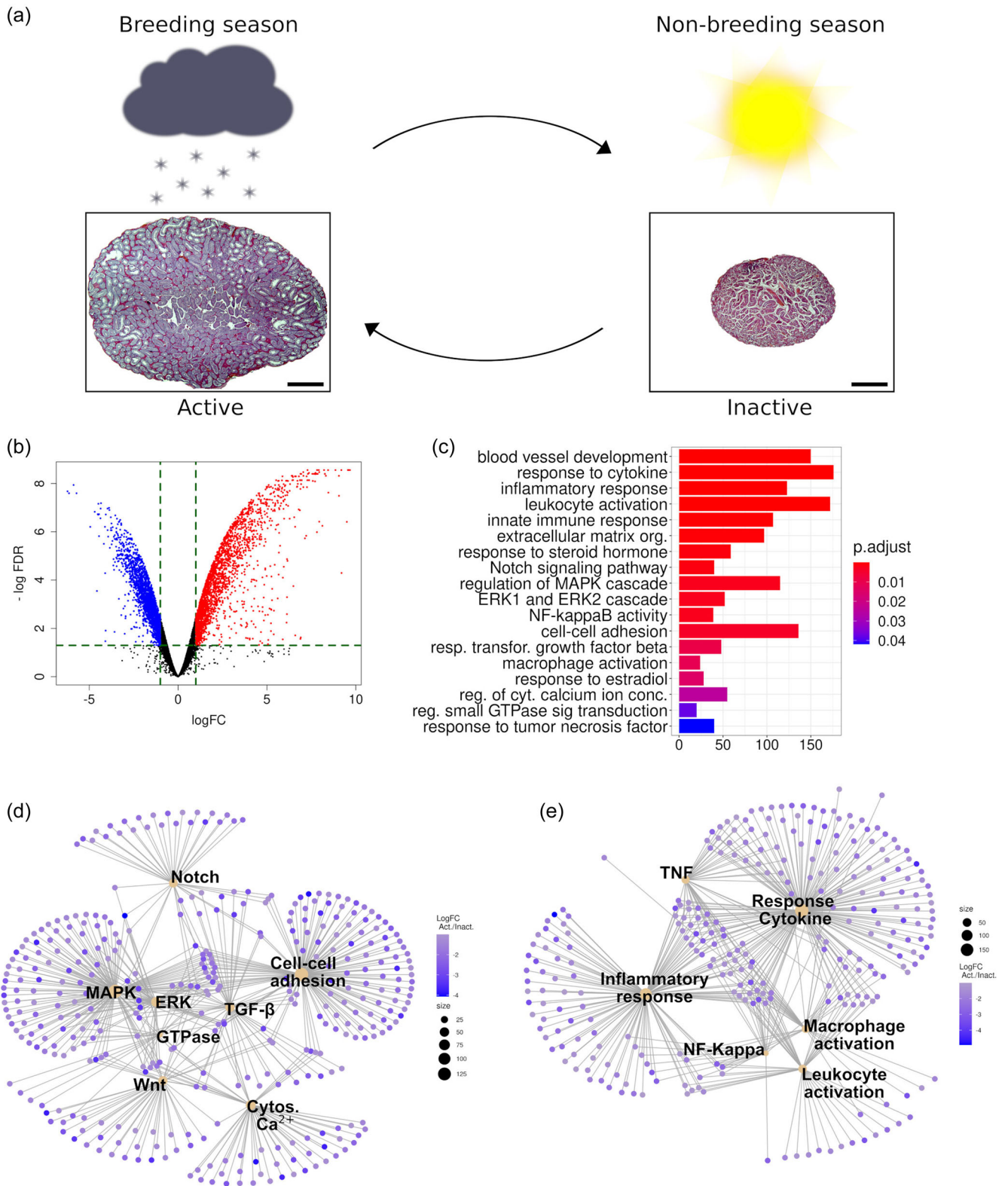


FIGURE 1 (See caption on next page)

shrink due to the germinative epithelium disorganization caused by a massive desquamation of live meiotic and post-meiotic germ cells (Figure 2a–c; Dadhich et al., 2010, 2013). Therefore, we also captured moles in April and generated transcriptomes from inactivating (regressing) testes. Multidimensional scaling plot showed that replicate samples of the same reproductive season clustered together, the inactivating samples being located between the active and the inactive ones. In this plot, the separation between active and inactivating testes was shorter than that between inactivating and inactive ones, confirming that we obtained transcriptomes corresponding to testes that were likely initiating the regression process (Figure 2d). Differential expression analysis between active and inactivating testes identified 452 DEGs, from which 207 were upregulated and 245 downregulated in the samples of the inactivating testes ($FDR < 0.05$ and $|\log_2FC| > 1$; Figure 2e; Supporting Information: Table S7), a number much smaller than that of DEGs identified between active and inactive testes (see above). From these 452 DEGs, 446 were also differentially expressed between active and inactive testes. Almost all genes found to be downregulated in one comparison (active/inactivating) were also downregulated in the other one (active/inactive), and the same happened with the upregulated genes (Figure 2f; see \log_2FC s in Supporting Information: Table S8). In general, the amplitude of the changes in gene expression observed in the comparison active/inactive was greater than that in the active/inactivating one (Figure 2f, note that the \log_2FC s vary between -5 and 9 in the first case (x-axis), and between -3 and 3 in the second one (y-axis); Supporting Information: Table S8). Accordingly, the magnitude of the expression changes in most genes ($|\log_2FC|$) was greater in the active/inactive comparison than in the active/inactivating one (red dots in Figure 2f; Supporting Information: Table S8). GO analysis using either all the DEGs or just the downregulated genes identified in the active/inactivating comparison revealed no significant enriched category. Contrarily, in the upregulated genes we found a significant enrichment ($Padjust < 0.05$) in a number of biological processes (Figure 2g; Supporting Information: Table S9), related to epithelium development, cell migration, wound healing and vasculogenesis (Figure 2g). We did not find any significantly enriched GO term associated to signaling pathways. So, we decided to search for DEGs between active and inactivating testes in the molecular pathways identified in the previous analysis (Figure 1e; Supporting Information: Table S4). We found 29 genes belonging to “MAPK cascade” (GO:0000165), 14 genes to the “ERK1

and ERK2 cascade” (GO:0070371), 11 to “response to transforming growth factor beta” (GO:0071559), 11 to “regulation of small GTPase mediated signal transduction” (GO:0051056; Supporting Information: Table S10), 7 to “regulation of cytosolic calcium ion concentration” (GO:0051480), and 6 to “Notch signaling pathway” (GO:0007219). In addition, we found 27 genes altered in the category “cell-cell adhesion” (GO:0098609). Gene-concept analysis using these data revealed an interacting network with many of these genes shared by several categories (Figure 2h). Altogether, these results suggest that the expression of genes belonging to several molecular pathways is altered at the beginning of testis regression, and that this alteration affects more genes (and probably more pathways) as the regression proceeds, thus ensuring the maintenance of the regressed status of the inactive testes of *T. occidentalis*. The MAPK/ERK1/2 pathway seems to play an essential role in this process.

2.3 | Transcriptomic analysis of early spermatogenesis in the regressed testis of *T. occidentalis*

We next investigate gene expression in the extant germ cells of the regressed testis. Consistent with our previous observations, double immunofluorescence for DMRT1, a marker of Sertoli and spermatogonial cells, and for DMC1, a marker for zygotene and early pachytene primary spermatocytes, revealed that spermatogonia maintain active proliferation in inactive testes and that a small number of spermatocytes reach the early pachytene stage (Figure 3a,b; Dadhich et al., 2011). Because of this, we decided to study the cell-specific expression profile of the early stages of spermatogenesis in active and inactive testes of the Iberian mole. For this, we used the gene expression signature of spermatogenic clusters reported by Hermann et al. (Hermann et al., 2018), assigning the genes we found to be differentially expressed between active and inactive testes to each of the early spermatogenic clusters, from undifferentiated spermatogonia to pachytene spermatocytes (Supporting Information: Table S11). Within these clusters, the number of downregulated genes increased as spermatogenesis progressed, being predominant at the pachytene stage (Figure 3c). However, this is probably a consequence of the much higher number of pachytene spermatocytes present in the active testis (see red cells in Figure 3a,b), rather than a reflection of actual changes in gene expression within cells. Biological theme comparison of downregulated genes in the inactive testes within these clusters

FIGURE 1 Transcriptomic analysis of seasonally active and inactive testes of *Talpa occidentalis*. Low magnification of hematoxylin and eosin-stained histological sections of seasonally active and inactive testes of the Iberian mole during the breeding (winter) and nonbreeding (summer) seasons (a). Note the pronounced reduction in testis size occurring during seasonal testis regression in this species. Scale bars represent 1 mm. (b) Volcano plot of the differential gene expression between active and inactive testes after normalization for different contents in germ cells. (c) Gene ontology analysis of the deregulated genes revealed a significant enrichment ($Padjust < 0.05$) in biological processes and molecular pathways associated to normal testicular functions. (d) Cnetplot of several significantly enriched molecular pathways identified in our GO analysis. (e) Gene-concept analysis of several significantly enriched GO terms associated with the activation of the immune system. In pictures (c–e), red color indicates downregulation and bluish color upregulation during testis regression. In figures (d, e), the size of sepia circles is proportional to the number of deregulated genes they represent.

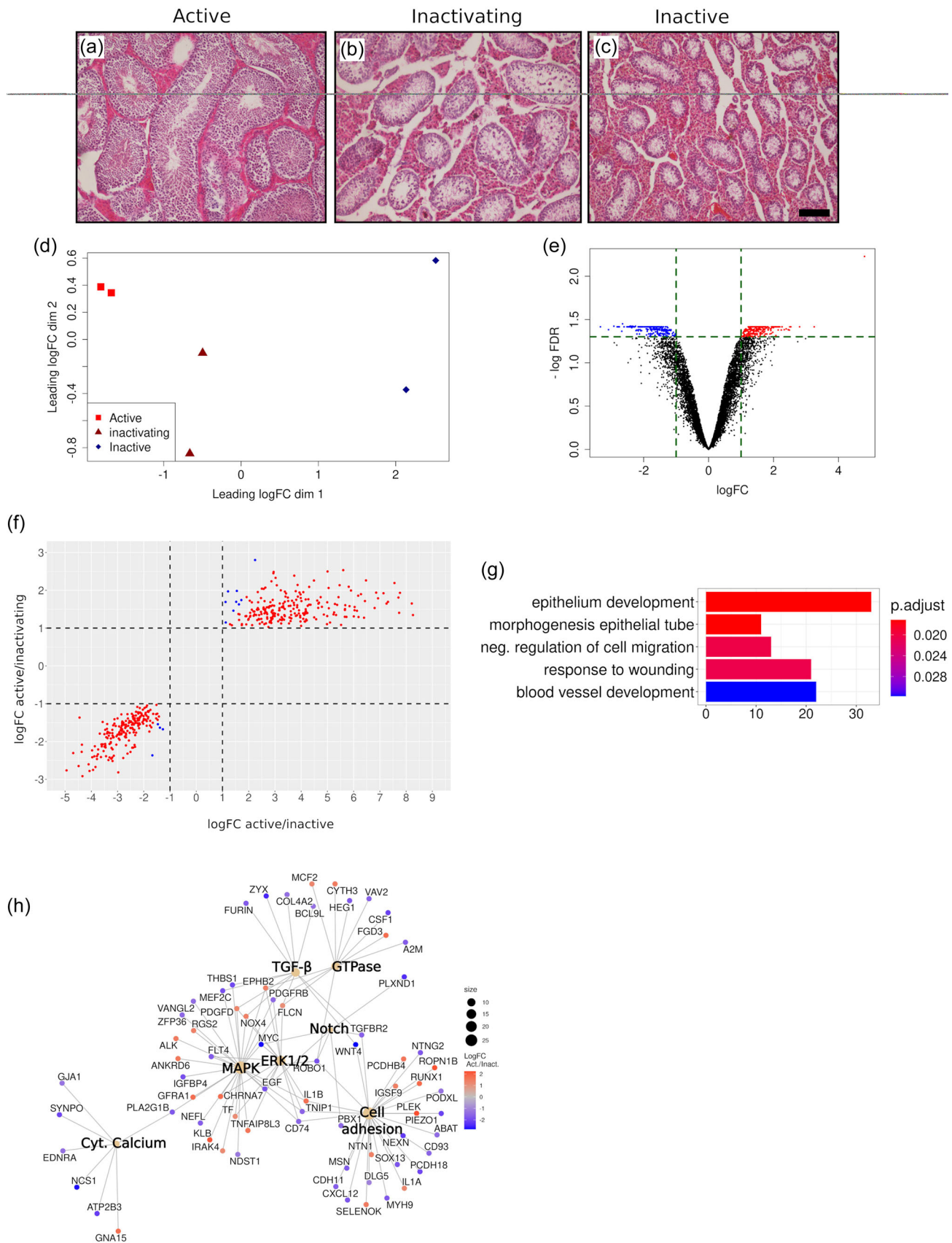


FIGURE 2 (See caption on next page)

(excluding the pachytene cluster) revealed in spermatogonial cells an enrichment of terms associated to “protein polyubiquitination” (GO:0000209), “covalent chromatin modification” (GO:0016569), “regulation of chromosome organization” (GO:0033044) and “DNA methylation” (GO:0006306). From the differentiated spermatogonia stage on, we identified GO categories associated to meiosis, including “nuclear chromosome segregation” (GO:0098813) and “meiotic nuclear division” (GO:0140013) among others (Figure 3d; Supporting Information: Table S12). As most of these latter biological processes are not completed in the inactive gonads, the differential expression detected for these genes is again probably a consequence of the different germ cell contents

of active and inactive testes. Biological theme comparison of upregulated genes showed an enrichment in general biological processes such as “cotranslational protein targeting to membrane” (GO:0006613), “protein localization to endoplasmic reticulum” (GO:0070972), and “cellular respiration” (GO:0045333) among others (Figure 3e; Supporting Information: Table S13). Overall, these results clearly show that polyubiquitination seems to be a function affected in spermatogonial cells during testis regression. However, our findings at later stages (early meiotic prophase) are less consistent as the detected alterations could probably be derived from the different germ cell contents of seasonally active and inactive testes of *T. occidentalis*.

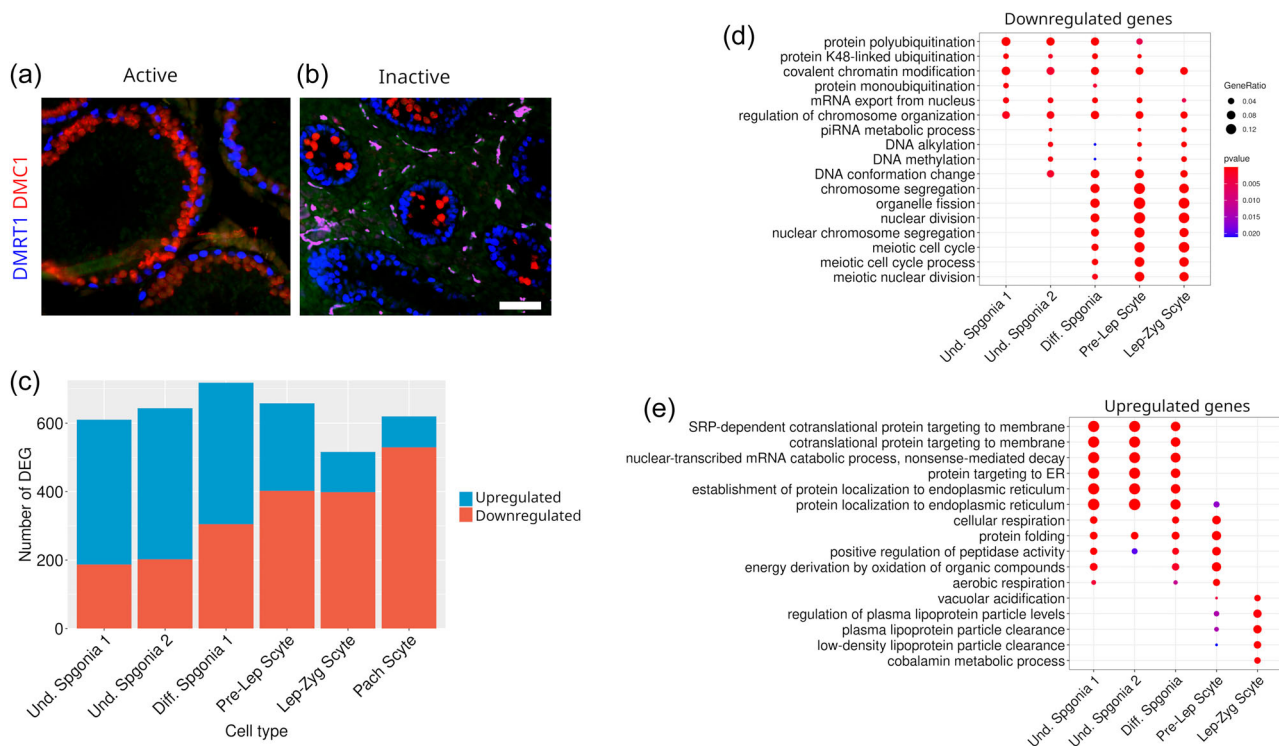


FIGURE 3 Transcriptomic analysis of early spermatogenesis in the inactive testis of *Talpa occidentalis*. Double immunofluorescence for DMRT1 (a marker for both Sertoli and spermatogonial cells) and for DMC1 (a marker for zygotene and early pachytene spermatocytes) in active (a) and inactive (b) testes. Note that the number of primary spermatocytes (red cells) is highly reduced in the inactive testis. (c) Number of genes predicted to be deregulated in each of the cell types of the early spermatogenic stages in the inactive testes of *T. occidentalis*. (d) Biological theme comparison of genes downregulated during testis regression in the early stages of spermatogenesis of the Iberian mole. (e) Biological theme comparison of genes upregulated during testis regression in the early stages of spermatogenesis of the Iberian mole. Scale bar in (b) represents 50 μm for (a, b). Original colors in (a, b) have been changed to make the figure accessible to color-blind readers.

FIGURE 2 Transcriptomic analysis of regressing (inactivating) testes of *Talpa occidentalis*. (a–c) Hematoxylin and eosin-stained histological sections of active (a), regressing (b), and inactive (c) testes of the Iberian mole. Note that seminiferous tubules of regressing testes have an intermediate size between those of active and inactive ones. (d) Multidimensional scaling plot of replicate samples of testes. Note that the regressing samples are placed between the active and inactive ones. (e) Volcano plot of the differentially expressed genes (DEGs) between active and regressing testes. (f) Log₂ fold change scatterplot representing the DEGs detected in the comparison between active and inactive testes against those observed in the comparison between active and regressing ones. (g) GO analysis of the deregulated genes identified in the comparison between active and regressing testes. (h) Gene-concept analysis of DEGs belonging to several molecular pathways. In pictures (e and h), red color indicates gene downregulation and bluish color upregulation during testis regression. In figure (h), the size of sepia circles is proportional to the number of deregulated genes they represent. Scale bar in (c) represents 100 μm for (a–c).

2.4 | Several biological processes are commonly affected in the regressed testes of both *T. occidentalis* and *M. duodecimcostatus*

We have recently reported the changes that the testicular transcriptome of the Mediterranean pine vole, *M. duodecimcostatus*, undergo during its seasonal reproductive cycle in southeastern Iberian Peninsula (Lao-Pérez et al., 2021). The inactive testes of this species show a clear difference with those of the Iberian mole: meiosis initiation is completely stopped, so that no zygotene or pachytene cells are present in the regressed seminiferous tubules. To explore the similarities of testicular regression between moles and voles, which are representative species of the Eulipotyphla and Rodentia orders, respectively, we decided to compare our testis transcriptomic datasets. We initially searched for genes that were either upregulated or downregulated in the regressed testes in both species ($FDR < 0.05$ and $|\log_2FC| > 1$), and identified 1529 genes, 900 of which were upregulated and 629 downregulated (Figure 4a,b and Supporting Information: Table S14). For these genes, we plotted the \log_2FC of one species against the other one and found a linear correlation between both sets of data (Figure 4c; Pearson correlation test, cor. coeff = 0.85; $p < 2.2 \times 10^{-16}$), showing that many alterations in gene expression occur during the testis regression process of the two species. As expected, GO analysis of downregulated genes revealed a significant enrichment ($Padjust < 0.05$) in a reduced number of biological processes related to spermatogenesis and sperm differentiation (Supporting Information: Table S15). In contrast, in the group of upregulated genes, we identified many categories that were also detected in our previous analyses (Figure 4d; Supporting Information: Table S16), including “cell-cell adhesion (GO:0098609; $Padjust = 1.7 \times 10^{-3}$)” and “extracellular matrix organization” (GO:0030198; $Padjust = 1.2 \times 10^{-5}$). This analysis also reported enriched GO terms associated to the same molecular pathways identified separately in both species, such as “regulation of MAPK cascade” (GO:0043408; $Padjust = 9.3 \times 10^{-6}$), “response to transforming growth factor beta” (GO:0071559; $Padjust = 3.9 \times 10^{-4}$), “ERK1 and ERK2 cascade” (GO:0070371; $Padjust = 3.8 \times 10^{-4}$), and “regulation of cytosolic calcium ion concentration” (GO:0051480; $Padjust = 2 \times 10^{-2}$) (Figure 4d; Supporting Information: Table S16). Gene-concept analysis revealed a complex interacting network with genes shared by several categories (Figure 4e). Moreover, our GO analysis also identified several enriched categories related to the activation of the immune system (Figure 4d; Supporting Information: Table S16), including “positive regulation of NF-kappaB transcription factor activity” (GO:0051092; $Padjust = 9 \times 10^{-4}$), “cytokine production” (GO:0001816; $Padjust = 7 \times 10^{-7}$), “macrophage activation” (GO:0042116; $Padjust = 2 \times 10^{-3}$), “response to tumor necrosis factor” (GO:0034612; 4×10^{-2}), and “leukocyte activation” (GO:0045321; $Padjust = 2 \times 10^{-7}$). Gene-concept analysis on these terms again revealed a cooperative network (Figure 4f), indicating that activation of the immune system is a common feature in the regressed testes of both species.

3 | DISCUSSION

We have previously reported the seasonal changes that the testes of *T. occidentalis* undergo at the histological, immunohistological, and hormonal level (Dadhich et al., 2010, 2011, 2013). To deepen in the molecular mechanisms underlying these changes, we have analyzed here the transcriptome of testes at different time points in the reproductive cycle of this species. We reported previously that, during the nonbreeding season, male moles have reduced levels of serum testosterone and regressed testes in which spermatogenesis is arrested, expression of cell adhesion molecules is disrupted and the BTB is not functional (Dadhich et al., 2013). Consistent with this, our transcriptome study shows that biological processes such as “cell-cell adhesion” and “cell junction assembly” as well as several molecular pathways including MAPK, ERK1/2, TGF- β , Cytosolic Ca^{2+} , PI3K, GTPase, and TNF (which operate in SCs and are necessary for spermatogenesis), and the dynamics of tight and adherens junctions forming the BTB, are altered in the inactive testes of *T. occidentalis*. The mitogen-activated protein kinases (MAPKs) comprise a family of regulators involved in the control of many physiological processes (Y. Sun et al., 2015). There are three classical subfamilies of MAPKs, (a) the extracellular signal-regulated kinases (ERKs), (b) the c-Jun N-terminal kinases (JNKs), and (c) the p38 MAPKs, all of which are known to regulate several aspects of the testicular function, including cell division and differentiation during spermatogenesis and junctional restructuring of the seminiferous epithelium (Ni et al., 2019; Q. Y. Sun et al., 1999; C.-H. Wong & Yan Cheng, 2005). The MAPK/ERK1/2 pathway plays essential roles in modulating cell adhesion and motility in several epithelia, including adhesion-mediated signaling (Howe et al., 2002), cytoskeleton dynamics (Stupack et al., 2000), and junction disassembly (Y. Wang et al., 2004). In the testis, the components of MAPK/ERK1/2 are found in SCs and all classes of germ cells in the seminiferous epithelium (C.-H. Wong & Yan Cheng, 2005), and regulates the formation of Sertoli-Sertoli and Sertoli-matrix anchoring junctions and the tight junction forming the BTB (Crépieux et al., 2001, 2002). This MAPK cascade also regulates the formation of ectoplasmic specialization (ES), structures that contribute to the adhesion between SCs at the BTB, and between Sertoli and developing spermatids at the adluminal compartment (Ni et al., 2019; Q. Y. Sun et al., 1999; C.-H. Wong & Yan Cheng, 2005). We found 132 and 52 genes belonging to the MAPK and ERK1/2 pathways, respectively, upregulated in the inactive mole testes (Supporting Information: Table S6, GO:0000165, and GO:0070371), and our gene-concept analysis showed that many of them are shared by these two pathways and by other processes, including cell junction assembly and regulation and cAMP-mediated signaling (Figure 1f). As mentioned above, MAPK can also act through the p38 MAPK cascade (Engelberg, 2004). This subfamily is activated by different pathways, including GTPases, usually resulting in inflammatory responses or apoptosis. Members of the p38 MAPK pathway have been found in SCs and elongate spermatids, and play a role in controlling cell junction dynamics in the seminiferous epithelium (C.-H. Wong & Yan Cheng, 2005). In SCs, this pathway

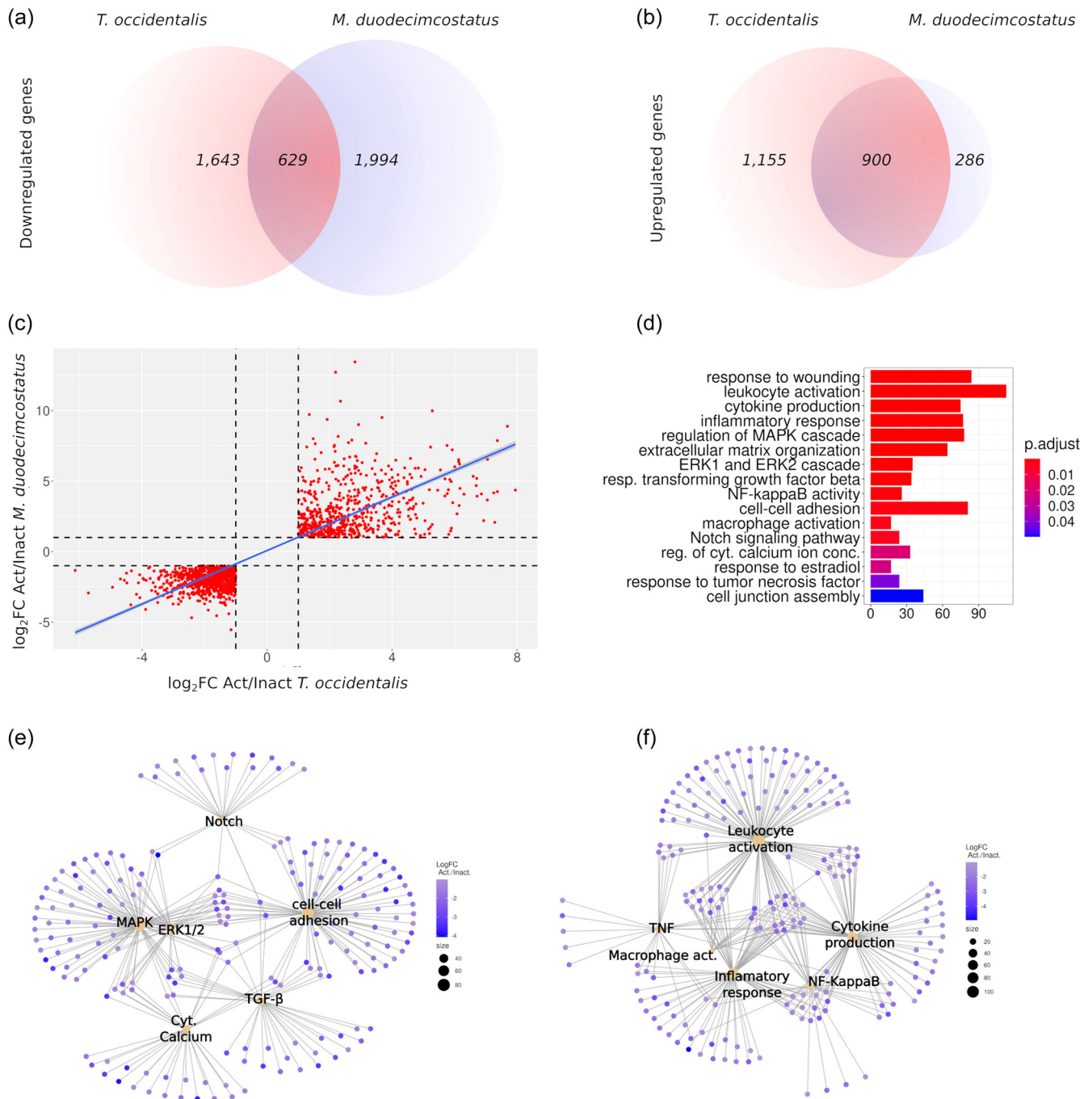


FIGURE 4 Biological functions altered in the inactive testes of both *Talpa occidentalis* and *Microtus duodecimcostatus*. (a,b) Venn-diagram representing the numbers of common genes downregulated (a) and upregulated (b) during testis regression. (c) \log_2 fold change scatterplot representing the differential gene expression between active and inactive testes of *T. occidentalis* against the same data from *M. duodecimcostatus*. (d) Gene ontology analysis of DEGs shared by both *T. occidentalis* and *M. duodecimcostatus*. (e) Gene-concept analysis of several significantly enriched GO terms associated with known molecular pathways acting in the testes of both species. (f) Gene-concept analysis of several significantly enriched GO terms associated with the activation of the immune response in both species. In pictures (e,f), red color indicates downregulation and bluish color upregulation of genes during testis regression, and the size of sepia circles is proportional to the number of deregulated genes they represent.

is activated in the presence of TGF- β 3, leading to disruption of the tight-junction proteins in the BTB (Lui et al., 2003a, 2003b; C. Wong et al., 2004). Our transcriptomic analysis also revealed that the TGF- β and the GTPase pathways are altered in the mole inactive testes

(Figure 1f; Supporting Table S4). The different MAPK cascades are likely to act in concert to regulate the BTB dynamics that facilitate germ cell migration throughout the seminiferous epithelium during the spermatogenic cycle (C.-H. Wong & Yan Cheng, 2005), and

several observations confirmed that these pathways are hormonally regulated. Testosterone can stimulate the MAPK/ERK signaling (Cheng et al., 2007; Fix et al., 2004) and low levels of this hormone, together with increased levels of TGF- β 3, leads to the loss of cell adhesion molecules in the seminiferous epithelium, a process that seems to be mediated by different MAPK cascades (Y. Wang et al., 2004; C.-H. Wong & Yan Cheng, 2005). In light of this knowledge, our current transcriptomic data strongly suggest that the reduced levels of testosterone that the Iberian mole undergoes during the inactive season lead to the activation of different MAPK signaling cascades in the testes, a fact that in concert with other molecular pathways, including GTPase, PI3-K and TGF- β signaling, deregulates the cell adhesion function in the seminiferous epithelium, leading to BTB disruption and spermatogenic arrest.

As mentioned above, seasonal testis regression implies the massive depletion of meiotic and postmeiotic germ cells of the testis. In *T. occidentalis*, this process occurs by desquamation of live, non-apoptotic germ cells. We found that most of the genes deregulated during this period do remain deregulated during the nonbreeding period, although at a less significant level. Among them, we found genes involved in the regulation of pathways that control cell adhesion such as MAPK, ERK1/2, GTPase, and TGF- β , indicating that deregulation of these pathways is likely to be also involved in the massive germ cell desquamation that accompanies seasonal testis regression in the mole.

A special immunological environment referred to as “immune privilege” operates in functional testes and protect germ cells from autoimmune attack. There are three main factors contributing to this immune privilege: (a) the existence of the BTB, which isolates meiotic and postmeiotic germ cells from the cells of the immune system, (b) the reduced capacity of the testicular macrophages to mount an inflammatory response, and (c) the production of anti-inflammatory cytokines by somatic cells (reviewed in (Fijak & Meinhardt, 2006; Li et al., 2012; Zhao et al., 2014). Our transcriptomic analysis revealed that categories related to immunological processes including “inflammatory response” “leukocyte activation” “macrophage activation” and “response to cytokine,” were altered in the inactive testes of *T. occidentalis*, as well as molecular pathways that regulate the immune system, such as NF-kappaB and TNF, denoting the activation of the immune system in the inactive testes of *T. occidentalis*. Under normal physiological conditions, testicular macrophages present a reduced capability to mount inflammatory responses and to produce cytokines, when compared with macrophages from other tissues. Our RNA-seq data revealed both the activation of the macrophage population and cytokine production in the inactive testes of the Iberian mole and that TNF and NF-KappaB, two molecular pathways involved in the regulation of inflammatory cytokines production (Hayden & Ghosh, 2014), operate in the inactive testis. Several studies evidence an immunosuppressive role of testosterone on different components of the immune system (Foo et al., 2017; Trigunaite et al., 2015), so that testicular testosterone induces a reduction of pro-inflammatory cytokines in macrophages (D'agostino et al., 1999). Taking all these observations into account, we suggest

that low levels of testosterone in the regressed testes of *T. occidentalis* may lead to the loss of the “immune privilege”, which is manifested by BTB permeation and increased cytokine production by the macrophage population (and perhaps other somatic cells). Altogether, these processes might contribute to maintain the quiescent status of the mole gonads during the nonbreeding period.

We also analyzed the expression profile of genes belonging to the genetic expression signature of early spermatogenic cell populations (Supporting Information: Table S11) and found that several biological processes are altered in the regressed testes of the Iberian mole, particularly protein ubiquitination at the spermatogonia stage (Figure 3d; Supporting Information: Tables S12 and S13). Ubiquitination is essential for the establishment of both spermatogonial stem cells and differentiating spermatogonia and it is also involved in the regulation of several key events during meiosis, including homologous recombination and sex chromosome silencing (Bose et al., 2014). Indeed, mutations in the ubiquitin specific protease 26 (USP26), which is expressed in Leydig cells and early spermatogonia (Wosnitzer et al., 2014), are associated with defective spermatogenesis and infertility in both human and mice (Paduch et al., 2005; Sakai et al., 2019). Testosterone supports spermatogenesis through three mechanisms: (a) maintaining the BTB integrity (Meng et al., 2011), (b) regulating SC-spermatid adhesion (Holdcraft & Braun, 2004), and (c) controlling the release of mature sperm (Holdcraft & Braun, 2004). All these actions are mediated by SCs, as germ cells do not express the androgen receptor (AR) and, thus, are not direct targets of testosterone. In this study, we reveal that gene expression seems to be altered in the spermatogonial cells of the inactive mole testes, although it is difficult to know whether this is caused either by the particular testicular environment of quiescent testes, in which both the BTB and the cell adhesion function are disrupted, or by currently unknown mechanisms directly affecting germ cell expression, or both.

Finally, we have compared the mole testicular transcriptomic data with those we recently reported for the Mediterranean pine vole. We found a large number of genes that are deregulated in the regressed testes of both species, with two remarkable coincidences: (1) many of these genes are involved in the control of cell adhesion (Figure 4b,c; Supporting Information: Tables S14 and S15) and, accordingly, molecular pathways such as MAPK, ERK1/2, TGF- β , GTPase, and TNF, which control cell junctions in the seminiferous epithelium, are deregulated in the two species; (2) we also found a shared set of genes involved in the regulation of the immune response. These coincidences are relevant if we consider that the inactive testes of these two species do not show identical features. For example, meiosis initiation by spermatogonia is completely abolished in the inactive testes of *M. duodecimcostatus* (Lao-Pérez et al., 2021), but not in those of *T. occidentalis*, where meiosis entry continues and spermatogenesis progresses until the early primary spermatocyte stages (Dadhich et al., 2010, 2013). Moreover, the inactive seminiferous tubules of *M. duodecimcostatus* remain adjacent to each other (Lao-Pérez et al., 2021), whereas those of *T. occidentalis* become widely separated from each other by intervening Leydig cells

(Dadhich et al., 2013; Lao-Pérez et al., 2021). Despite these differences, here we report that two important testicular functions, cell adhesion, and immune response, are altered in the inactive testes of these two species, indicating that these are common molecular mechanisms operating in the regressed testes of seasonally breeding mammals.

4 | MATERIAL AND METHODS

4.1 | Animals

Six adult males of Iberian mole were captured alive in poplar groves near the locality of Chauchina (Granada province, south-eastern Spain) at three key stages of the reproductive cycle, using the methods developed in our laboratory (Barrionuevo et al., 2004). Two animals were captured in December (reproductive season), two more in April (transition period when testis regression occurs), and the last two in July (nonreproductive season). Animals were dissected, and the testes were removed under sterile conditions. The gonads were weighed, and frozen in liquid nitrogen for mRNA purification and further RNA-seq studies. A 5-mm thick slice of one of the testis of every animal was fixed in 50 volumes of 4% paraformaldehyde overnight at 4°C, embedded in paraffin, and processed for histology and immunofluorescence. Animals were captured with the permission of the Andalusian environmental authorities (Consejería de Agricultura, Pesca y Medio Ambiente) following the guidelines and approval of both the Ethical Committee for Animal Experimentation of the University of Granada and the Andalusian Council of Agriculture and Fisheries and Rural Development (Registration number: 450-19131; June 16th, 2014).

4.2 | Immunofluorescence

Testis sections were deparaffinized and incubated with primary antibodies overnight, washed, incubated with suitable conjugated secondary antibodies at room temperature for 1 h and counter-stained with 4',6-diamino-2-phenylindol (DAPI). We used a Nikon Eclipse Ti microscope equipped with a Nikon DS-Fi1c digital camera (Nikon Corporation) to take photomicrographs. In negative controls, the primary antibody was omitted. The primary antibodies used were goat-anti-DMC1 (Santa Cruz Biotechnology, CA, sc-8973; 1:100) and rabbit-anti-DMRT1 (a kind gift from Sivana Guioli, 1:200). The secondary antibodies used were: green—donkey anti-rabbit IgG Alexa Fluor 488 (A32790); red—donkey anti-goat IgG Alexa Fluor 555 (A32816).

4.3 | RNA-seq

Total RNA was isolated from both testes of the two males captured in every time point using the Qiagen RNeasy Midi kit following the manufacturer's instructions. After successfully passing quality check

(RIN value > 7 measured in a Bioanalyzer, Agilent Technologies), the RNAs samples were paired-end sequenced separately in an Illumina HiSeq. 2500 platform at the Max Planck Institute for Molecular Genetics facilities in Berlin, Germany.

4.4 | Bioinformatics

The quality of the resulting sequencing reads was assessed using FastQC (<http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/>). The RNA-seq reads were mapped to the recently published genome of *T. occidentalis* (Real et al., 2020) with the *align* and *featureCounts* function from the R subread package (Liao et al., 2019). As mentioned above, most meiotic and postmeiotic germ cell types (from primary spermatocyte to spermatozoa) are exclusive of the active testes, being completely absent in inactive ones. Thus, many genes expressed in germ cells would appear as overexpressed in sexually active testes as such results would not reflect changes in gene expression but only differences in cell contents between active and inactive testes. Such an over-representation of germ-cell-specific transcripts in active testes would mask changes in gene expression of somatic cells, which do exert the control of the spermatogenic cycle, in particular SCs. Hence, to normalize the data and focus on the study of gene expression in somatic cells, we decided to remove transcripts expressed in germ cells from the General Feature Format (GFF) file of the Iberian mole that we have recently generated (Real et al., 2020). For this, we used previously published cell signatures in single-cell RNA sequencing studies (scRNA-seq) (Green et al., 2018; Hermann et al., 2018), as described in Lao-Pérez et al. (2021). We removed all genes included in clusters 1–13 and 16 from the Hermann et al. (2018) study, belonging to different germ cell types, and those from spermatogonia, spermatocyte, round spermatid, and elongating spermatid from the Green et al. (2018) one. After doing this, the number of genes analyzed decreased from 13,474 (Supporting Information: Table S1) to 8300 (Supporting Information: Table S3). Analysis of differential gene expression was performed with edgeR (Robinson et al., 2010). Genes were filtered by expression levels with the *filterByExpr* function, and the total number of reads per sample was normalized with the *calcNormFactors* function. Genes were considered to be differentially expressed at $\text{P}_{\text{adj}} < 0.05$ and $|\log_{2} \text{FC}| > 1$. GO analysis was performed with the *enrich* GO function of the *clusterProfiler* bioconductor package (Yu et al., 2012). General terms and terms not related with testicular functions were not displayed.

AUTHOR CONTRIBUTIONS

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in ArrayExpress at <https://www.ebi.ac.uk/arrayexpress/>, reference number E-MTAB-10836.

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