Publishable summary-Final report

Summary:

The study focuses in particular on mechanism of germ cell migration during early postnatal and later during spermatogenesis through the testicular development. Early postnatal migration comprises of gonocytes migrating to the basal part of seminiferous tubule to produce spermatogonia. Second migratory event take place throughout spermatogenesis where passage of differentiating spermatocytes through blood-testis-barrier (BTB). The study focuses on mechanism of germ cell migration at these two time points conditional knockout mouse model (cKO) for CAR (the coxsackie virus and adenovirus receptor (CAR; official symbol CXADR). The overall objectives of this study are as follows 1) to investigate the role of CAR in germ cell migration across blood-testis-barrier (BTB) and early postnatal migration of gonocytes to basal membrane. 2) Effects of cytotoxic agents on BTB integrity. 3) Exploring the possible interacting partner that helps CAR in this process. We knocked out CAR at two time point, the adult and early postnatal period. Characterization of the morphological and functional aspect of testicular function was carried out. We found that in adult downregulation of CAR does not affect the morphology of the testis, the mice were fertile. BTB was intact and was fully functional as determined both by use of a biotin labelled tracer and Lanthanum tracer. These tracers were retained in the interstitial space and are restricted to enter the seminiferous tubules due to the presence of functional BTB. The distribution of other junctional proteins like occludin, ZO-1, JAMA, claudin 3 remained normal as judged by immunofluorescence staining. The ultrastructural examination by transmission electron microscopy revealed that the junctions appeared normal. When CAR is knocked out in the early postnatal mice, there was efficient floxing of CAR gene and CAR protein level was also decreased. We do not observe any morphological differences in the testis of 6-day-old mice as compared to control. The testis from these mice showed normal appearance of differentiating spermatocytes at postnatal day 24. The search for interacting partner for CAR showed that JAML protein was found to be highly expressed in the adult testis. Also JAML interacts with CAR in the testes based on immunoprecipitation experiments. The integrity of the BTB and the expression and localization of BTB components were analyzed as part of collaborative project. The result showed that the effect of doxorubicin is age dependent and the early pubertal mice showed mislocalization of the junctional proteins. In conclusion these results indicate that CAR might not have a direct role in the migration of the germ cells at these two critical periods of testicular development. The other explanation could be that a small amount of remaining CAR protein is enough to help migrating germ cells. The model can be further improved by using a Sertoli cell CAR knock out in order to knock out CAR efficiently.

Description of project context and objectives:

The first event of migration take place immediately after birth that constitute movement of prospermatogonia from the lumen of the seminiferous tubules toward the peripheral region where the spermatogonial stem cells find their niche. Second migration comprise of actively dividing mitotic spermatocytes across blood-testis-barrier (BTB) towards the lumen of

seminiferous tubules during active spermatogenesis. The BTB is constituted largely by inter-Sertoli cell tight junctions. It divides the seminiferous tubule into basal and adluminal compartment. Migration through BTB has been extensively studied and the maintenance of the barrier integrity during migration has been greatly emphasised. One of the widely accepted theories suggests a transit intermediate compartment between the adluminal help germ cell migration through BTB based on morphological evidence. Several members of junctional protein families are implicated in the movement of germ cells among these are claudins, occludin and CTX family members like Coxsackie- and Adenovirus Receptor (CAR) and Junction Adhesion Molecules (JAMs). CAR and JAM-C have been shown to be crucial for normal heart development and germ cell differentiation, respectively. Interestingly JAML and CAR interaction is important in *leukocyte migration across endothelial- and/or epithelial barriers* and we have also shown that JAML is expressed in the cultured Sertoli cells. In this contest similar interactions might be important in germ cell migration. In testis CAR is expressed in high amounts during early postnatal period (up to day 12) and then its expression becomes stage dependent with highest concentration at the stage VII-IX where the germ cells are migrating through BTB. This expression pattern thus suggests a role in these two migration events.

The overall objective of this study was to explore participation of CTX family of proteins in particular CAR in facilitation of migration process at two major time point of testicular development that is early postnatal migration of prospermatogonia to the peripheral region of seminiferous tubules and later migration of spermatocytes towards lumen of seminiferous tubules across blood-testis-barrier (BTB). We also explored the interacting partner and downstream signalling pathways of CAR contributing to its functions in testis. As a collaborative endeavour we have also studied BTB junctional component like Pg1 and its correlation with protection of testis against toxic effects of cytotoxic agents. In this contest the effects of cytotoxic agents on formation and integrity of BTB is explored.

Work performed and results

The work is divided into three packages. A brief summary of all the packages and main result achieved in each package is described below

1. Workpackage I: CAR and germ cell migration

The objectives of the package 1 were to assess the role of CAR in the two germ cell migratory events occurring during postnatal testicular development. First being the early postnatal migration event of gonocytes from the centre of lumen towards the peripheral region where gonocytes or prospermatogonia are differentiated to spermatogonia and germ stem cell finds their niche. The second migration event is initiated during adulthood where the differentiating spermatocytes move from basal to the adluminal compartment of the seminiferous tubules to become differentiated sperms. This process involves trans-epithelial migration through BTB and ensures continual spermatogenesis. Initial characterization of second phase of migration that is in the adult animal was done in year 1 and is completed in the 2nd year. This part largely focuses on characterization of early postnatal gonocyte migration and the embryonic testicular development after E13.5. **Three major finding are reported in this package. 1.**

Adult CAR cKO testis displayed normal spermatogenesis and fertility. Distribution of other junctional proteins and BTB integrity was unaffected. 2. CAR down regulation in the early postnatal period does not effect gonocyte migration and later spermatogenesis, followed up to the age of P24 in these mice. 3. The embryonic development of testis after E13.5 is not affected by the CAR down regulation. The conclusions from these results however should be treated with caution as minimal amounts of CAR protein is still present that might be enough for the migration of germ cell at the phases analyzed. This notion is also supported by our data on normal appearance and expression of other junctional proteins like claudin 3 & 11, occludin and JAMA that are not changed as compensatory mechanism. (Fig.1 & 2)

2. **Work package II: BTB dynamics: effects of cytotoxic agents**

The main objective of this package was characterizing the intermediate compartment that exists at the BTB during the transit of spermatocytes to the luminal compartment of the seminiferous tubules**.** The nature of the compartment is dynamic and sort lived appearing at stage VIII of the seminiferous tubules as suggest by the studies by our group. The approach was to construct the 3D images of this compartment by utilizing several microscopies like Dual beam microscope and TEM together with confocal microscope and Laser microdissection to finally analyze component of the compartments. Our analyses were limited basically to electron microscope and use of confocal microscope. Due to the failure of the application of both laser microdissection and dual beam electron microscopy to characterize the intermediate compartment alternative approaches were utilized and the dynamics of BTB is studied instead. Three model systems were designed in form of collaborative projects as outlined below. A) Using CAR cKO mouse model to study the effect of absence of CAR on BTB formation and maintenance. This approach is extensively described in work package. B) Analysis of ontogeny and expression of drug transporter Pg1 an integral components of BTB in young, early pubertal and adult rats. C) Age dependent effects of cytotoxic drug (doxorubicin) on BTB formation and integrity in rats.

The above mentioned studies are designed as collaborative projects and are still ongoing. The results obtained to date from these studies can be summarized as follows. **a) Pg1 is integral part of BTB and is found to be age dependently regulated with increasing expression from PP6 and onwards. The level of cytotoxicity of doxorubicin is negatively correlated with the expression level of the Pg1. PP6 was found to have lowest expression of Pg1, hence maximum toxicity effect by doxorubicin is observed. b) The expression and localization of junctional proteins is changed in early pubertal rats after 3 and 6 days of doxorubicin treatment. No such effects were observed in immature and adult rat testis**.

3. Work Package III: Interacting partners of CAR

The main objectives of this package were to characterize interacting partners and the downstream signaling of CAR in testis. For this purpose primary Sertoli cell cultures were established to utilize it as *in vitro* model system to analyze cells from CAR cKO testis. The aim was to knock out CAR in Sertoli cell culture and study effects on downstream signaling of CAR. CAR has been shown to interact with other junctional protein and forms both hemophilic and heterophilic dimers. Such interactions were observed with CAR-CAR, CAR-

JAMA and CAR-JAML. In particular CAR-JAML interaction is important in trans-endothelial migration and could be important in testis too in trans-epithelial migration ongoing at BTB. In order to analyze interaction of CAR and JAML constructs were generated and immunoprecipitation experiment of CAR and JAML from testis was performed. Several commercially available antibodies for JAML were screened for this purpose. The main results achieved to date are **a) Establishment of the *in vitro* primary Sertoli cell culture from 14-day-old mice. The Sertoli cell culture from control and CAR cKO mice has been set up and CAR has been down regulated by adding Tamoxifen. RNA isolation and microarray experiments are on the way. b) CAR-JAML interaction has been shown to be present in testis by immunoprecipitation experiments.**

The potential impact (including the socio-economic impact and the wider societal implications of the project so far) and the main dissemination activities and exploitation of results

The study is an attempt to explore the complex mechanism of germ cell migration during the two critical periods of testicular development and spermatogenesis using a transgenic model for one of the important junctional protein CAR that has role in heart, pancreas and . Failure to migrate at these two steps results in the testicular dysgenesis syndrome that includes cryptochidism and Sertoli cell only syndrome and infertility. The phenomenon has been explained by many theories and a number of players required for this process has been identified a few with functional evidence. The study exploits the use of transgenic technology to explore the in vivo function of a protein. The other aspect of the project that is study of dynamics of BTB also revealed important information regarding harmful effects of anti cancer drug on BTB formation. Other studies has concentrated on the direct toxic effect of this drug on germ cells our report points toward the effect of this drug on Sertoli cell function in form of its effects on BTB disturbances. Thus, suggesting the use of this drug with caution in early pubertal boys.

Apart from presentation of these result in European Testis workshop May 2010 the results of the cKO model characterization has been submitted for publication and is in review process to date. The above results on testicular phenotype characterization in young and adult mice are now being complied as a manuscript. The fellow has also substantially participated in the cytotoxic effects on BTB dynamic the results of this study are also being complied as manuscript. Taken together the Marie Curie fellowship has helped the fellow in developing her skills and greatly increased scientific understanding.

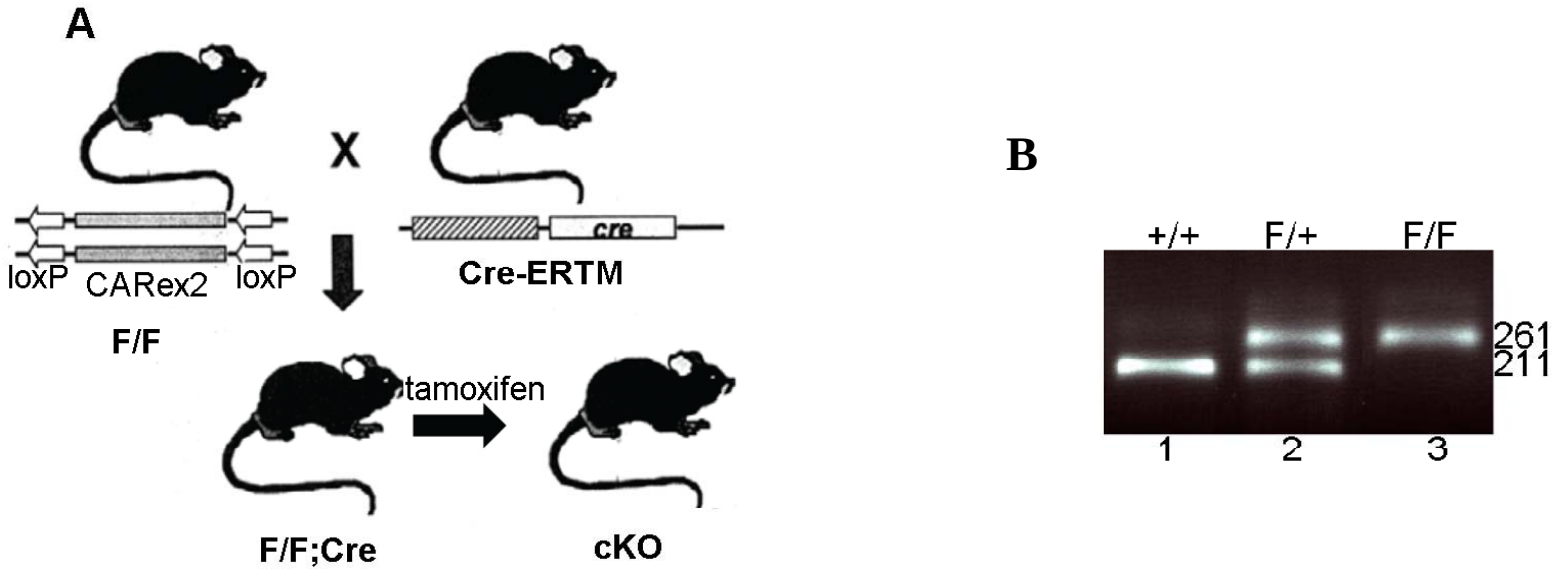


Figure 1 A.The gene targeting strategy to creat CAR cKO mice. The lox p inserted mice were crossed with Cre-ERTM mice. In order to activate cre animals are injected with tamoxifen for 5 days. B represents genotypying with wild type (+/+), heterzygotes (F/+) and homozygotes (F/F)

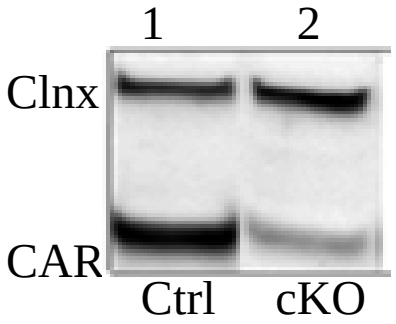


Figure 2A: Expression of CAR is downregulated in cKO mice as compare to control. Calnexin is used as loading control

Fig 2B control

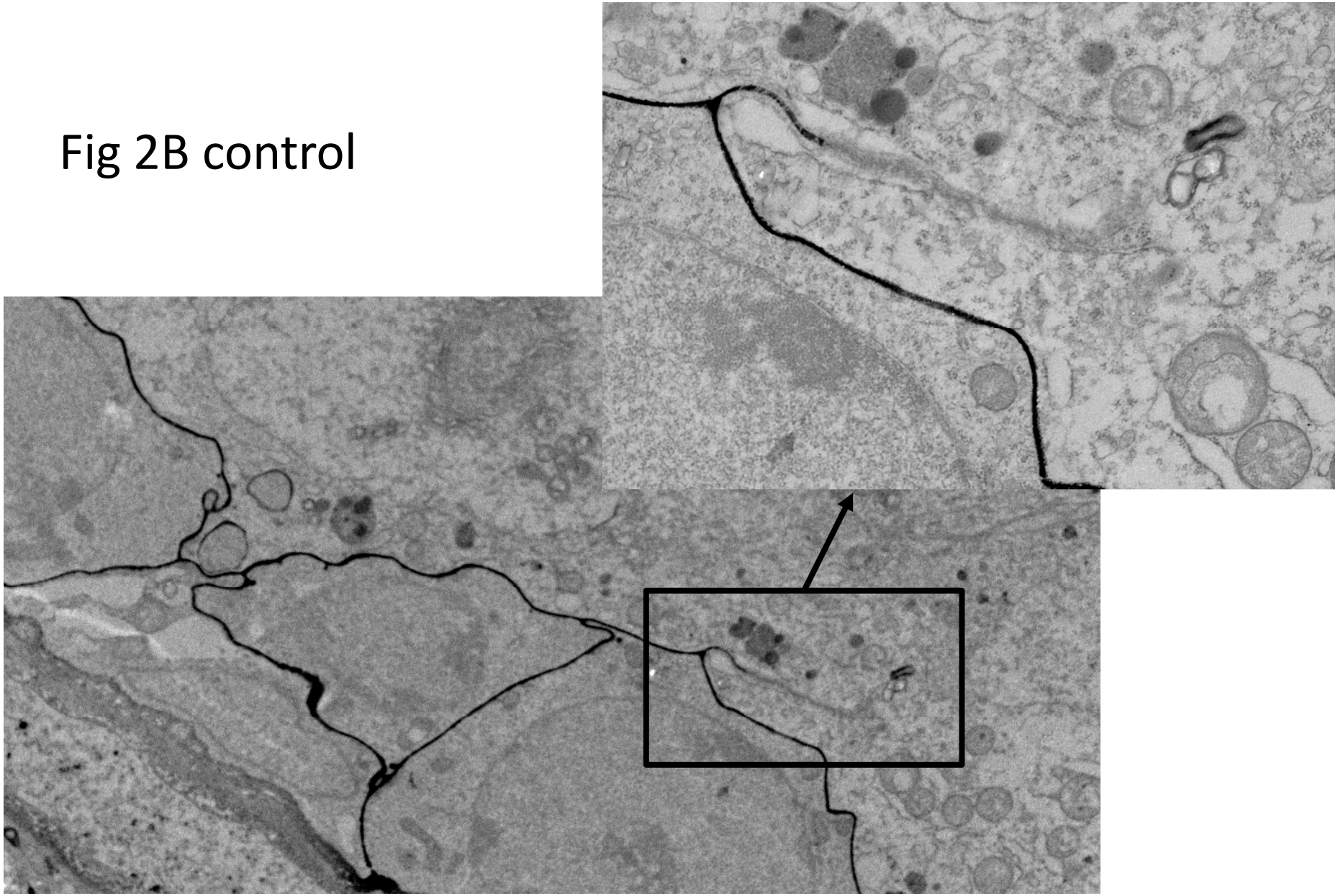
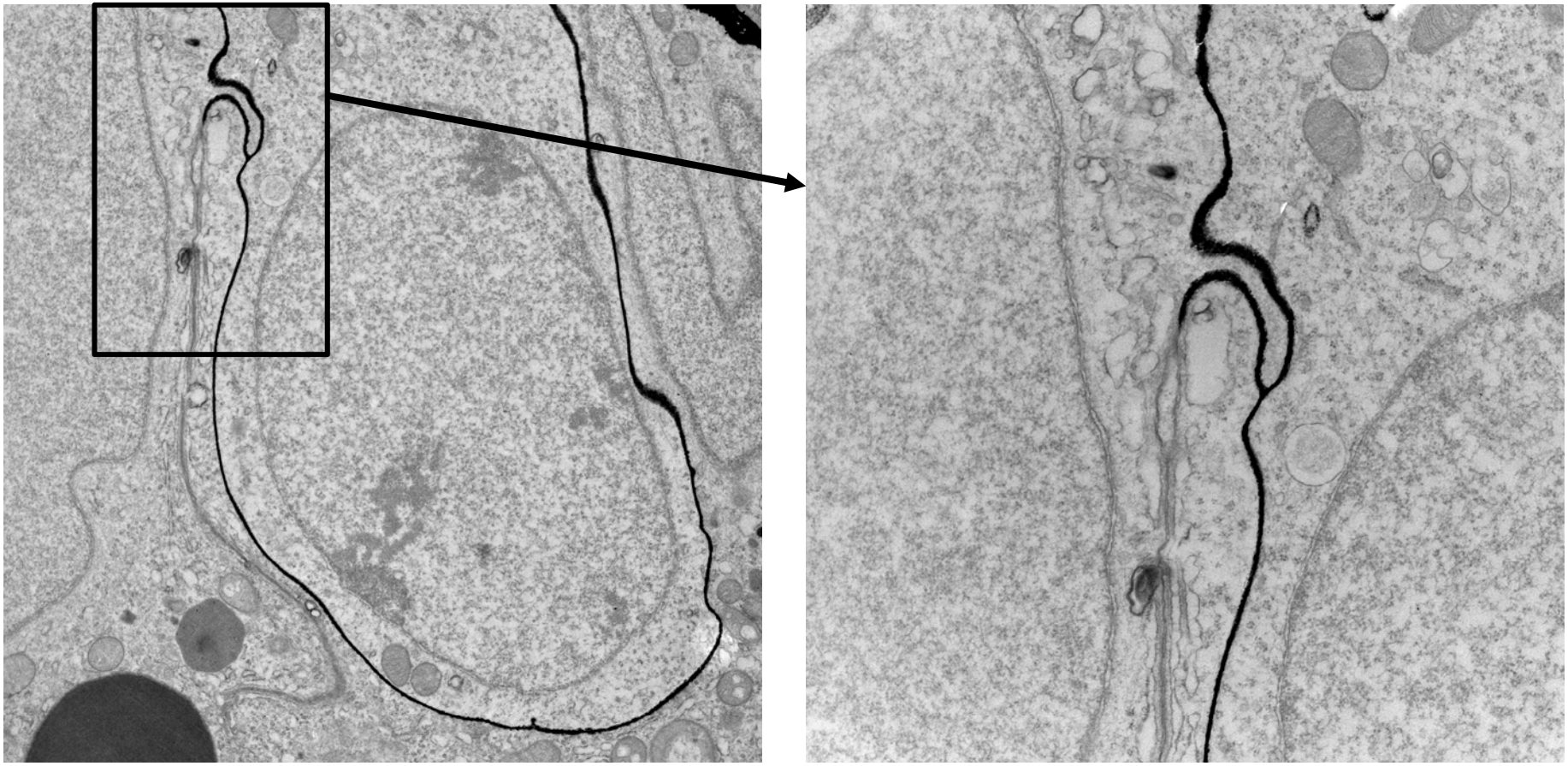


Fig 2 B. Lanthanum tracer experiment showing intact BTB in control mice.

Fig 2B CAR cKO



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