Developmental expression patterns of chemokines CXCL11, CXCL12 and their receptor CXCR7 in testes of common marmoset and human testes

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| Complete List of Authors: | Westernströer, Birgit; University of Münster, Centre of Reproductive Medicine and Andrology  
Langenstroth, Daniel; University of Münster, Centre of Reproductive Medicine and Andrology  
Kliesch, Sabine; University Clinic Muenster, Department of Clinical Andrology  
Troppmann, Britta; University of Münster, Centre of Reproductive Medicine and Andrology  
Redmann, Klaus; University of Münster, Centre of Reproductive Medicine and Andrology  
Macdonald, Joni; MRC/University of Edinburgh, Centre for Reproductive Health  
Mitchell, Rod; MRC/University of Edinburgh, Centre for Reproductive Health  
Wistuba, Joachim; University of Münster, Centre of Reproductive Medicine and Andrology  
Schlatt, Stefan; Univ Münster, CeRA  
Neuhaus, Nina; University of Münster, Centre of Reproductive Medicine and Andrology |
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Resubmission of the revised manuscript (CTR-14-0494.R1) entitled ‘Developmental expression patterns of the chemokines CXCL11, CXCL12 and its receptor CXCR7 in common marmoset and human testes’.

Dear Prof. Unsicker, dear Prof. Sutovsky,

thank you for the second review of our manuscript. We are delighted to see that the paper is close to acceptance. We revised the manuscript in accordance to the suggestions by referee 1. We herewith submit a slightly revised version with some changes to the discussion.

We are grateful that you consider our work for publication in Cell and Tissue Research.

Yours sincerely,

Prof. Dr. Stefan Schlatt
Response to Reviewer

Reviewer: 1

I re-examined the revised version of the manuscript. The authors have addressed all of my concerns and the manuscript has been modified accordingly.

Response: We thank the reviewer for this positive assessment.

1) However, I have noted that the same group has recently published a paper on the profiling of CXCR4 and CXCR7 during murine testis development (Westernstroer et al. 2014). The expression pattern of CXCR7 in the murine germ cells as well as the expression profile of CXCL12 (assessed by qRT-PCR) is not perfectly in line with data obtained on monkey and human testis. CXCR7 expression is retained in more differentiated murine germ cells, and murine CXCL12 is stable up to 21 postnatal day and increases after 37 postnatal days. In my opinion, the authors should discuss and comment on this in more details (page 21, lane 354).

Response (lines 322 – 333, 362 – 367): We expanded the discussion as suggested.
Title: Developmental expression patterns of the chemokines CXCL11, CXCL12 and its receptor CXCR7 in common marmoset and human testes

Authors: Birgit Westernströer, Daniel Langenstroth, Sabine Kliesch, Britta Troppmann, Klaus Redmann, Joni Macdonald, Rod Mitchell, Joachim Wistuba, Stefan Schlatt, Nina Neuhaus

1 Centre of Reproductive Medicine and Andrology, CeRA, Albert-Schweizer-Campus 1, Building D11, 48149 Münster, Germany

2 Department of Clinical Andrology, Centre of Reproductive Medicine and Andrology, CeRA, Albert-Schweitzer-Campus 1, Building D11, 48149 Münster, Germany

3 MRC/University of Edinburgh Centre for Reproductive Health, The Queen’s Medical Research Institute, 47 Little France Crescent, Edinburgh, EH164TJ, Scotland, UK

Corresponding author: Prof. Dr. Stefan Schlatt

Centre of Reproductive Medicine and Andrology

Albert-Schweizer-Campus 1 (D11)

48149 Muenster, Germany

Email: Stefan.Schlatt@ukmuenster.de

Key words: CXCR7, CXCL12, CXCL11, primate testes, testicular development
Abstract

The chemokine receptor CXCR7 interacts with the chemokines CXCL11 and CXCL12. During development this ligand receptor system (C-X-C) provokes cell type-specific responses in terms of migration, adhesion or ligand sequestration. It is active in zebrafish and rodents but no data are available for its presence or function in primate testes. Real-time quantitative PCR was performed in the monkeys to detect CXCL11, CXCL12 and CXCR7. At the protein level CXCL12 and CXCR7 were localized in marmoset (Callitrix jacchus) testes whereas CXCR7 patterns were determined for various stages of human testes. Morphometry and flow cytometry were applied to quantify CXCR7-positive cells in monkeys. Transcript levels and protein expression of CXCR7 were detectable throughout testicular development. In both species CXCR7 protein expression was restricted to premeiotic germ cells. In immature marmoset testes 69.9 % ± 9% of the total germ cell population were labelled for CXCR7 whereas in the adult 4.7% ± 2.7% were positive for CXCR7. CXCL12 mRNA was detectable in all developmental stages of marmosets. The CXCL12 protein was exclusively localized to Sertoli cells. This pattern of CXCL12/CXCR7 indicates its involvement in regulatory processes that may concert the interaction between undifferentiated germ cells and Sertoli cells.
Introduction:

The (C-X-C-motif) chemokine receptor type 7 (CXCR7) is a deorphanized G-protein-coupled seven-span transmembrane receptor which binds the chemokines CXCL12 (also called stromal-derived factor-1, SDF-1) and CXCL11 (also called interferon-inducible T cell α-chemoattractant, I-TAC) (Balabanian et al. 2005; Burns et al. 2006). Whereas CXCL12 is expressed under homeostatic conditions to modulate immune surveillance and development, the expression of CXCL11 is upregulated upon stimuli and therefore belongs to the inflammatory chemokines. In general, CXCL11 production is stimulated by interferons in a variety of cells, including leukocytes, fibroblasts and endothelial cells (reviewed in: Sánchez-Martin et al. 2013) and therefore supports the massive cell infiltration in several inflamed tissues. The chemokines CXCL12 and CXCL11 bind also to the conventional chemokine receptors CXCR4 and CXCR3, respectively (Balabanian et al. 2005). However, CXCR7 binds CXCL12 and CXCL11 with higher affinity and belongs to the group of atypical chemokine receptors (ACKRs). Interestingly, ACKRs are unable to couple to G-proteins following ligand binding and therefore fail to induce the full spectrum of classical G-protein-coupled chemokine receptor signalling and cellular responses (Comerford et al. 2007; Ulvmar et al. 2011; Cancellieri et al. 2013; Nibbs et al. 2013). Instead, recent studies have demonstrated that CXCR7 can signal through versatile adapter molecules and acts as an endogenous β-arrestin-biased signalling receptor (Rajagopal et al. 2010). In addition, CXCR7 may serve as a co-receptor for CXCR4 and therefore enhances the classical CXCL12-mediated G-protein signalling (Levoye et al. 2009; Luker et al. 2009).

In particular, the action of CXCR7 has been shown to be important for several steps in developing organs (Sánchez-Martin et al. 2013). Interestingly, expression of CXCR7 was detected in various cell types. For instance, expression was found on the cell surface of mesenchymal stem cells (Liu et al. 2010) as well as on renal progenitor cells (Mazzinghi et al. 2008). However, in the primordium of the zebrafish, CXCR7 expression was restricted to
the somatic environment. More specifically, it has been demonstrated that a CXCL12 gradient in the somatic tissue facilitates the directed migration of CXCR4-positive primordial germ cells (PGCs) towards the future gonad (Boldajipour et al. 2008; Mahabaleshwar et al. 2008; Staton et al. 2011; Mahabaleshwar et al. 2012). In this regard, expression of CXCR7 is of particular importance as it acts as a scavenger for CXCL12 and is therefore responsible for the generation of a CXCL12 gradient (Boldajipour et al. 2008; Mahabaleshwar et al. 2008; Staton et al. 2011; Mahabaleshwar et al. 2012). In addition, the CXCL12/CXCR4 interaction is also of importance for PGC migration in mice, suggesting a conserved mechanism (Ara et al. 2003; Molyneaux et al. 2003).

Moreover, recent studies in juvenile as well as in adult mouse and human testes have revealed that CXCL12 is secreted by Sertoli cells and showed that this chemokine is required for the migration and maintenance of undifferentiated spermatogonia within the stem cell niche (Gilbert et al. 2009; Payne et al. 2010; Kanatsu-Shinohara et al. 2012; Yang et al. 2013). Transcripts of Cxcr7 have been detected in developing mouse testes (Westernströer et al. 2014) as well in testes obtained from adult rats and humans (Eva et al. 1993; McIver et al. 2013). In addition, the cellular localization of Cxcr7 in developing mouse testes has been shown in premeiotic germ cells suggesting that Cxcr7 may be involved in the regulation of the spermatogonial population (Westernströer et al. 2014).

Regarding testicular development and testicular stem cell physiology, the common marmoset monkey is similar to the human (Li et al. 2005; Mitchell et al. 2008; Albert et al. 2010; Albert et al. 2012; Lin et al. 2012; McKinnell et al. 2013) and is therefore an appropriate animal model to study the CXCR7 axis in the developing testis further. Therefore, we used immature and adult human testicular tissues and employed the marmoset monkey (Callitrix jacchus) as a non-human primate animal model in order to elucidate the expression pattern of CXCR7 and its ligands at distinct stages of testicular development.
The primary aim of the present study was to investigate mRNA expression levels of CXCL12, CXCL11 and CXCR7 in marmoset monkeys during four different phases of testicular development. Based on these results, the second aim was to identify and to characterize the cellular localization of CXCR7 protein expression during testicular development in the marmoset and human testis.

Material and Methods

Animals, collection and processing of testicular tissue

All experimental procedures were performed in compliance with the German Federal Law on the Care and Use of Laboratory Animals (animal license No. 84-02.05.20.12.0. 18, LANUV NRW, Germany). Common marmoset monkeys, Callitrix jacchus, were maintained at the institutional breeding facilities under standardized conditions. Marmosets were fed with pellets from Altromin (Lage, Germany) with daily supplement of fresh vegetables and fruits.

In addition, they had unlimited access to tap water and were kept under 12h light / 12h darkness cycles.

Monkeys (n = 4 / group) were sacrificed under anaesthesia (ketamine/xylazine solution in saline; i.m.; 0.1 ml per 100 gram body weight) at the age of 16, 32, 52 and > 80 weeks post-partum (wpp). Testes were dissected and one half was snap-frozen for subsequent RNA expression analysis, whereas the remaining tissue was fixed in Bouin’s solution for 24 hrs, transferred into 70% EtOH and embedded in paraffin for immunohistochemical analyses.

For flow cytometric analysis, adult marmosets (n = 3) were sacrificed as outlined above. Testes were dissected and transferred into pre-cooled minimum essential medium-alpha (MEM-α, Life Technologies, Gibco, Darmstadt, Germany), followed by enzymatic digestion for flow cytometry.
Human testicular tissues and preparation for histological analyses

Human adult testicular tissues were obtained from patients attending the department of Clinical Andrology due to treatment of testicular tumours. Of such patients testicular tissue which was assessed to be unaffected by the tumour and with complete spermatogenesis was used in this study (Ethics Committee of the Medical Faculty of Muenster and the State Medical Board no. 2006-588-f-S). Status of spermatogenesis was assessed according to Bergmann and Kliesch (2010).

Tissue was fixed in 4% paraformaldehyde (PFA) for 24 hrs, before it was transferred into an increasing ethanol series (30%, 50% and 70%) and embedded in paraffin. For histological evaluation, 3 µm sections were prepared and were stained using periodic acid Schiff’s reagent (PAS staining) followed by haematoxylin counterstaining as previously described (Brinkworth et al. 1995). Based on the histological analysis, testicular tissues (n = 2) with normal spermatogenesis were selected for this study.

In addition, human testicular tissues (age: 2 weeks and 6 months) from the Centre for Reproductive Health at the University of Edinburgh were used. Testes were obtained at autopsy with consent of their legal guardian from boys who died from various causes (excluding reproductive and endocrine abnormalities). Testes were fixed in 10% Neutral Buffered Formalin (NBF) for at least 24 hrs and transferred into 70% EtOH for processing.

Immunofluorescence on testicular tissue

Paraffin was removed using Pro Taqs Clear (Pro Taqs Clear, Quartett Immunodiagnostika & Biotechnologie, Berlin, Germany) and sections were rehydrated in a decreasing ethanol series. After washing with phosphate-buffered saline (PBS), sections were incubated with 5% (w/v) bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 20 min at room
temperature (RT). Primary antibodies against CXCR7 (rabbit polyclonal anti-CXCR7, Abcam, dilution of 1:400, Cambridge, UK) and MAGE-A4 (mouse anti-MAGE-A4, provided by Prof. G. C. Spagnoli from the University Hospital of Basel Switzerland, dilution of 1:20) were applied to the sections and sections were incubated in a humid chamber at 4°C for 1 hr. Following two washing steps in PBS, the appropriate Alexa fluor 488-linked or 546-linked secondary antibodies, diluted in TBS/5% BSA, were applied for 45 min at RT in the dark. Incubation with non-specific immunoglobulin G (rabbit IgG and mouse IgG, dilution of 1:1000) was used as negative control. Cells were counterstained with Hoechst (Sigma-Aldrich, Steinheim, Germany).

**Immunohistochemistry on testicular tissue**

Sections were dewaxed and rehydrated as described above. After rinsing with distilled water and TBS, non-specific peroxidases were blocked with 3% (v/v) H$_2$O$_2$ for 15 min at RT. In order to block non-specific binding sites, sections were incubated with 5% (w/v) bovine serum albumin (BSA) for 20 min at RT. Subsequently, primary antibodies against CXCR7 (rabbit polyclonal anti-CXCR7, Abcam, dilution of 1:400, Cambridge, UK) and CXCL12 (mouse monoclonal anti-CXCL12, R&D system, dilution of 1:50, Wiesbaden, Germany) were applied and sections were incubated overnight in a humid chamber at 4°C. Incubation with corresponding non-specific immunoglobulin G (rabbit IgG or mouse IgG, respectively) antibodies served as negative control. After three washing times with TBS, sections were incubated with biotin conjugated secondary antibodies and subsequently labeled with a streptavidin-conjugated horseradish peroxidase (Sigma-Aldrich, Steinheim, Germany). Staining was visualized using 3,3’-diaminobenzidine as chromogen (Sigma-Aldrich, Steinheim, Germany), and haematoxylin as counterstain.
For morphometric evaluation of marmoset testicular tissues, 4-µm sections were taken at 40-µm intervals. In tissue sections from infantile marmosets, a total of 100 seminiferous tubules per animal (n = 3) were analyzed to determine labeling indices of CXCR7-positive germ cells. In accordance with previous publications germ cell types were categorized into the following three groups, cells with no observable contact with the basement membrane were classified as gonocytes, those with some point of contact as pre-spermatogonia and those with full contact with the basement membrane as spermatogonia (Sharpe et al. 2003b). Additionally, the percentages of CXCR7-positive and negative gonocytes, pre-spermatogonia and spermatogonia were determined.

RNA isolation and relative gene expression analysis

RNA extraction was performed using the miRNeasy Kit (Qiagen, Hilden, Germany) and genomic DNA was removed by DNase treatment. cDNA was generated with the iScript cDNA Synthesis Kit (Bio-Rad, Munich, Germany) using 200 ng of total RNA. Specific primers were designed using Primer Express® 3.0 Software (Life Technologies, Darmstadt, Germany). Optimal primer concentrations, primer specificity and PCR efficiency were evaluated following the Power SYBR® Green PCR User Guide (Life Technologies, Darmstadt, Germany). Primer sequences are summarized in Table 1. For quantitative real-time PCR (qRT-PCR) analyses, cDNA was diluted 1:10 and 1.5 µl were used for each 15 µl PCR reaction with Power SYBR® Green Mastermix (Life Technologies, Darmstadt, Germany). The PCR programme consisted of initial steps of activation and denaturation which were run once for 10 min at 95°C, followed by 40 cycles of denaturation (15 sec at 95°C), annealing and elongation (1 min at 60°C). qRT-PCRs were run on the StepOnePlus™ (Life Technologies, Darmstadt, Germany) and were subsequently analysed using the
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StepOne™ software 2.2 (Life Technologies, Darmstadt, Germany). To calculate relative expression levels of CXCR7 (n = 4 / group), CXCL12 (n = 4 / group) and CXCL11 (n = 4 / group) in marmoset testes, the $2^{-\Delta\Delta CT}$ method (Livak et al. 2001) was applied, using TOP1 as reference gene. Each sample was measured in duplicate.

**Enzymatic digestion and flow cytometry of testicular cells**

Single-cell suspension of testicular cells was prepared by sequential enzymatic digestion. The seminiferous tubules were digested in MEM-α with 1 mg/mL collagenase I (Sigma Aldrich, Steinheim, Germany) in a water bath at 37°C for 40 min. The digestion was stopped by adding MEM-α containing 10% fetal bovine serum (FBS, Life Technologies, PAA, Darmstadt, Germany). Afterwards, the seminiferous tubular fragments were isolated from interstitial cells by sedimentation at unit gravity and the seminiferous tubular fragments were further digested with 1 mg/mL collagenase I, 2.2 mg/mL DNase I (Sigma Aldrich, Steinheim, Germany) and 0.5 mg/mL hyaluronidase (Sigma Aldrich, Steinheim, Germany) in MEM-α until a single-cell suspension was achieved. Finally, the single-cell suspension was centrifuged (1500 rpm, 4 min), the supernatant was removed and enzymatic digestion was stopped with FBS.

For haematoxylin staining, testicular single cells were fixed on poly L-lysine coverslips with 4% PFA for 15 min. After rinsing with PBS, cells were counterstained with haematoxylin at RT for 1 min and afterwards analyzed.

For flow cytometric analysis, single-cell suspensions were filtered through a 30 µm mesh and washed with 5% (w/v) goat serum. Subsequently, cells were collected by centrifugation (1500 rpm, 5 min), the supernatant was removed and cells were incubated with the primary antibody against CXCR7 (rabbit polyclonal anti-CXCR7, Abcam, dilution of 1:200, Cambridge, UK) on ice for 30 min. Incubation with the corresponding non-specific
immunoglobulin G (rabbit IgG, dilution of 1:2000) served as negative control. Following two washing steps in 5% (w/v) goat serum, cells were incubated on ice with an Alexa fluor 488-linked secondary antibody (goat anti-rabbit Alexa fluor 488, Life Technologies, Darmstadt, Germany, diluted in 5% goat serum for 30 min. Finally, stained cells were analysed using a BD FACSaria II (Becton Dickinson).

**Immunofluorescence staining of testicular single cells**

Fixed testicular single cells on poly L-lysine coverslips were rinsed with PBS. Cells were then incubated with 5% (w/v) BSA at RT for 30 min. Subsequently, primary antibodies against CXCR7 (rabbit polyclonal anti-CXCR7, Abcam, dilution of 1:400, Cambridge, UK) were applied and cells were incubated in a humid chamber at 4 °C for 1 hr. Following two washing steps in PBS, the appropriate Alexa fluor 546-linked secondary antibody, diluted in TBS/5% BSA, was applied for 30 min at RT in the dark. Incubation with non-specific immunoglobulin G (rabbit IgG, dilution of 1:2000) was performed as negative control. Cells were counterstained with Hoechst (Sigma-Aldrich, Steinheim, Germany).

**Histological sample evaluations and image acquisition**

Samples were analyzed using an Olympus BX61 microscope (Melville, NY, USA) with an attached Retiga 400R camera (QImaging, Burnaby, BC, Canada). All images were acquired digitally using cellSens imaging software (Olympus, Münster, Germany) and were processed using Adobe Photoshop CS2 9.0 (Adobe Systems, CA, USA).
Statistical analysis

Statistical analyses were performed using Graph Pad Prism 5 (Graph Pad Software, USA).

For analysis of qRT-PCR results the non-parametric Kruskal-Wallis test was performed followed by the Dunn’s multiple comparison test. For morphometric analyses the Student’s t-test was applied. For these two tests, significant differences between the groups are marked with *P<0.05.

Significant differences between the testis weights were determined using the “One way analysis of variance” followed by the “Tukey’s multiple comparison test”. Groups marked with different letters (a, b, c, d) are significantly different from each other (P<0.001).
Results

Testicular development, testis growth and expression pattern of CXCL12, CXCL11 and CXCR7 in postnatal marmoset testes.

Testicular tissues from four phases of testicular development (16 wpp, 32 wpp, 52 wpp and > 80 wpp) were selected to characterize the expression pattern of CXCR7, CXCL12 and CXCL11 in the marmoset monkey.

Histological examination of testicular tissues of marmoset monkeys at the age of 16 wpp (Fig. 1a) revealed the presence of gonocytes, pre-spermatogonia and spermatogonia. At the age of 32 wpp, differentiated germ cells up to spermatocytes were detected in seminiferous tubules of marmoset monkeys (Fig. 1b). At the age of 52 wpp (Fig. 1c), round as well as elongated spermatids were observed in the seminiferous tubules. Finally, spermatogenesis was completed in the seminiferous tubules of marmoset monkeys at the age of > 80 wpp (Fig. 1d). In these age groups, testis weights (Fig. 1e) increased significantly from 36.4 ± 6.3 mg (16 wpp) via 181.9 ± 35.7 mg (32 wpp) and 376.7 ± 72.0 mg (52 wpp) to 481.9 ± 36.5 mg (> 80 wpp).

Relative expression levels of CXCR7 (n = 4 / group), CXCL12 (n = 4 / group) and CXCL11 (n = 4 / group) during postnatal testis development in the marmoset were determined using qRT-PCR analysis. Expression of CXCL12 was high in marmoset testes at the age of 16 wpp (0.214445 ± 0.112887) and then decreased significantly (0.088170 ± 0.012248; 2.4-fold; 52 wpp) during testicular development (Fig. 1f). Compared to CXCL12, expression of CXCL11 was 56-fold (16 wpp), 76-fold (32 wpp), 21-fold (52 wpp) and 23-fold lower (> 80 wpp) and did not change significantly during postnatal testis development (Fig. 1g). Expression of the receptor CXCR7 (Fig. 1h) was high in marmoset testes at the age of 16 wpp (0.368448 ± 0.094921) and decreased significantly (0.092540 ± 0.013916; 4-fold; > 80 wpp) during postnatal development.
CXCL12 and CXCR7 protein expression in postnatal marmoset testes

Immunohistochemistry was performed to determine the expression of the homeostatic chemokine CXCL12 and the corresponding receptor CXCR7 during postnatal testis development in marmoset monkeys.

In the testes of marmoset monkeys at the age of 16 wpp (Fig. 2a), 32 wpp (Fig. 2c, d), 52 wpp (Fig. 2e, f) and > 80 wpp (Fig. 2g, h), CXCL12 was exclusively localized in the cytoplasm of Sertoli cells. In contrast, expression of CXCR7 was restricted to the germ cell population. More specifically, in marmoset testes at the age of 16 wpp the expression of CXCR7 was found in a subset of gonocytes (Fig. 3c), pre-spermatogonia (Fig. 3e) and spermatogonia (Fig. 3g). Furthermore, morphometric analyses revealed that the CXCR7 labelling index of the entire germ cell population was 69.9 ± 9.0% (Fig. 3i), which comprised CXCR7-positive gonocytes (Fig. 3c), pre-spermatogonia (Fig. 3f) and spermatogonia (Fig. 3h) with the indices of 36.8 ± 13.7%, 18.0 ± 4.1% and 15.1 ± 9.8%, respectively (Fig. 3i).

Double immunofluorescence stainings (Fig. 4a-l) revealed subpopulations of MAGE-A4+/CXCR7+ and MAGE-A4+/CXCR7+ gonocytes (Fig. 4d, e, f), pre-spermatogonia (Fig. 4g, h, i) and spermatogonia (Fig. 4j, k, l). However, subsets of MAGE-A4+/CXCR7- pre-spermatogonia (Fig. 4g, h, i) were only rarely observed in tubules of marmoset testes at the age of 16 wpp.

During postnatal development CXCR7 expression persisted in premeitoic germ cells located at the basement membrane (Fig. 5a-f). CXCR7-positive A_pale (Fig. 5b, d) and A_dark (Fig. 5f) spermatogonia were identified by morphological evaluation in marmoset monkeys at the age of 32 wpp, 52 wpp and > 80 wpp.

Furthermore, adult marmoset testes (n = 3) were enzymatically digested and isolated single cells were subjected to haematoxylin staining which showed cell types with different sizes and morphology (Fig. 5g). Subsequent, flow cytometric assessment revealed expression of
CXCR7 on the cell surface of 4.7 ± 2.7% of cells in the single cell suspension obtained from adult marmoset testes (Table S2) and CXCR7 protein expression was confirmed by immunofluorescence staining of cells fixed on poly L-lysine slides (Fig. 5h). The qualitative observations of CXCR7-positive cells following immunofluorescence staining were consistent with the data obtained by flow cytometry.

CXCR7 protein profiles in postnatal human testes

A panel of rare postnatal human testis samples (2 weeks, 6 months and adult) was immunohistochemically stained and revealed that a subset of gonocytes, pre-spermatogonia as well as spermatogonia was positive for CXCR7 in immature human testes (Fig. 6a-c). Moreover, the expression of CXCR7 in adult human testes (Fig. 6d) was found in premeiotic germ cells at the basement membrane.

Discussion

We have illustrated a schematic drawing of the localization patterns of CXCL12 and CXCR7 which are pointing to a crucial role of this chemokine/receptor pair in the developing primate testis (Fig. 7). Interestingly, transcript levels of CXCL12 were constitutively expressed and immunohistochemical analyses further demonstrated protein expression of CXCL12 in the cytoplasm of Sertoli cells at all stages of postnatal testicular development which indicated a non-maturation-dependent expression of the homeostatic chemokine CXCL12 in the developing marmoset testis. After birth, the highest number of Sertoli cells per testis is established at around 4 month of age. Afterwards, the Sertoli cell population decreases and after 8 month of age Sertoli cells become morphologically mature (e.g. establishment of tight-junctions between adjacent Sertoli cells, formation of lumen in the seminiferous tubules) in
marmoset testes (Sharpe et al. 2003a; Li et al. 2005). We therefore assume this phenomenon to explain the significantly decreased transcript levels of CXCL12 in the developing marmoset testis. The Sertoli cell-specific expression pattern of CXCL12 described in this study is in line with previous mouse (Payne et al. 2010; Yang et al. 2013, Westernströer et al. 2014) and human data (Gilbert et al. 2009). Here we describe for marmosets a decline of CXCL12 from week 16-52 (prepuberty to postpuberty). This mRNA pattern is different from that previously observed in mice. The mRNA levels of Cxcl12 in developing mouse testes remained constant from birth until day 21 and then increased until adulthood (Westernströer et al. 2014). The two methods for quantification of mRNA are different (mouse: luciferase based normalization; marmoset: reference gene based normalization) and the reported levels can therefore not be easily compared. However in mice and marmosets transcripts for CXCL12 are detectable and appear to be modified throughout pubertal development. Current results therefore suggest that the action of CXCL12 is evolutionarily conserved. Functions of CXCL12 have been described to be organ or tissue specific. This includes a role for cell proliferation or survival (Kortesidis et al. 2005; Burns et al. 2006; Mazzinghi et al. 2008), differentiation (Lazarini et al. 2003), adhesion (Burns et al. 2006; Mazzinghi et al. 2008) and the regulation of cell migration (Bhakta et al. 2006; Mazzinghi et al. 2008). Regarding germ cells, CXCL12 has been shown to be secreted by the somatic environment thereby facilitating the colonization of the gonads by PGCs during mouse embryonic development (Doitsidou et al. 2002; Ara et al. 2003; Molyneaux et al. 2003; Dambly-Chaudiere et al. 2007; Boldajipour et al. 2008). Furthermore, recent studies in neonatal and adult mice revealed that CXCL12 is required for maintenance of spermatogonial stem cells (SSCs) within their niches as well as for the homing and colonization process of SSCs into their niches following germ cell transplantation assays (Payne et al. 2010; Kanatsu-Shinohara et al. 2012; Yang et al. 2013). Moreover, we previously reported significantly increased transcript levels of Cxcl12 following germ cell
depletion in mice and suggested that Cxcl12 may stimulate the spermatogenic repopulation of the seminiferous tubules during testicular recovery (Westernströer et al. 2014).

Whereas inflammatory chemokines (e.g. the C-X-C chemokine CXCL11) are mainly involved in the recruitment of leukocytes to inflamed tissue sites, homeostatic chemokines (e.g. the C-X-C chemokine CXCL12) are constitutively expressed in organs and have important functions in stem cell homing, tissue homeostasis and repair. Indications for testicular inflammation (e.g. massive cell infiltration by neutrophils, lymphocytes, macrophages and mast cells in the interstitium) (Guazzone et al. 2009) were not seen in the analyzed testes. Moreover, in contrast to CXCL12, only low transcript levels of CXCL11 were detected in developing marmoset testes which confirmed that inflammatory C-X-C chemokines, especially CXCL11 as a secondary ligand for CXCR7, is likely not of functional relevance for healthy marmoset testes.

Transcript levels of CXCR7 were also constitutively expressed and immunohistochemical analyses further demonstrated that the atypical chemokine receptor CXCR7 is exclusively expressed by premeiotic germ cells (gonocytes, pre-spermatogonia, spermatogonia including A_pale and A_dark spermatogonia) throughout various phases of postnatal testicular development in the marmoset and the human testis. In agreement with these data we found that Cxcr7 protein can be detected in gonocytes as well as in Sall4-positive undifferentiated spermatogonia of the developing mouse testis (Westernströer et al. 2014). Additionally, Cxcr7 was also expressed on differentiated germ cells, spermatids and interstitial cells. These results reveal an evolutionarily conserved expression pattern of CXCR7 in premeiotic germ cells in the mouse and both analysed primate species (Westernströer et al. 2014). The morphometric evaluation in immature marmoset testes has shown additionally that approximately 80% of migrating germ cells (gonocytes and pre-spermatogonia) express CXCR7. In general, recent studies in marmoset monkeys indicated that the migration of gonocytes most likely begins postnatally between birth and 4 months of age (Rune et al. 2014).
Interestingly, several MAGE-A4+/CXCR7+ gonocytes were observed, whereas subsets of MAGE-A4+/CXCR7+ pre-spermatogonia were only rarely seen in seminiferous tubules, suggesting that CXCR7 may be involved in migration and/or adhesion of germ cells in immature marmoset testes. In general, migration of centrally located germ cells into the basal part of the seminiferous epithelium is a cellular process critical for germ cell survival and establishment of A\text{dark} and A\text{pale} spermatogonia which represent the reserve and active SSC pool in adulthood in marmoset and human testis (McGuinness and Orth, 1992; Orth et al. 2000). Moreover, our morphometric evaluation in immature marmoset testes has shown that around 50% of the spermatogonia at the basement membrane express CXCR7. Furthermore, we demonstrated that A\text{dark} and A\text{pale} spermatogonia express CXCR7 in marmoset testes at the age of 32 wpp, 52 wpp and > 80 wpp. Therefore, we assume different roles for the atypical chemokine receptor CXCR7 in the developing marmoset testes. Tissue- and cell type-specific functions of CXCR7 have been described and depend on the cell type and tissue. This includes a role for cell survival or adhesion as described for example for in the breast cancer cell line MCF-7 (Burns et al. 2006) as well as in renal multipotent progenitor cells of the human kidney (Mazzinghi et al. 2008).

In addition, using HEK-293 and MDA-MB-231 cells, it has been demonstrated that receptor heterodimerization between CXCR4 and CXCR7 may enhance CXCL12-induced cell migration (Levoye et al. 2009; Decaillot et al. 2011). Regarding the localization of CXCR4 in developing mammalian testes, different expression patterns have been shown for mouse and human (Gilbert et al. 2009; Yoon et al. 2009; Kanatsu-Shinohara et al. 2012; Yang et al. 2013, McIver et al. 2013). Whilst expression of CXCR4 was found in gonocytes, undifferentiated spermatogonia, Sertoli cells and interstitial cells of neonatal and adult mouse testes (Yoon et al. 2009; Kanatsu-Shinohara et al. 2012; Yang et al. 2013), CXCR4 protein was detected throughout the seminiferous tubules of adult human testes (Gilbert et al. 2009).
So far, the expression profile and the role of CXCR4 in developing marmoset testes remain unknown. Nevertheless, receptor heterodimerization between CXCR4 and CXCR7 throughout testicular development in primate testes cannot be excluded but needs to be investigated in further studies.

In conclusion, we show that CXCL12/CXCR7 network is present in primate testes. The localization pattern suggests its involvement during migration, survival or adhesion in premeiotic germ cells.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figures:

**Fig. 1** Marmoset monkey testes after birth - age-related changes in histology, weight and transcript levels. Micrographs showing PAS stained tissue sections of marmoset testes at the age of 16 wpp (wpp = weeks post partum; a), 32 wpp (b), 52 wpp (c) and > 80 wpp (d). Gonocytes (arrowhead), pre-spermatogonia (white arrow) and spermatogonia (black arrow) are the predominant germ cells in marmoset testes at the age of 16 wpp (a). Meiotic cells (primary spermatocytes) were present in the seminiferous tubules of marmosets at the age of 32 wpp (b), whilst germ cells up to the stage of elongated spermatids were detected in the seminiferous epithelium of marmoset monkeys at the age of 52 wpp (c). Finally, spermatogenesis was complete in all marmosets at the age of > 80 wpp (d). Scale bars represent 50 µm. Changes in testis weight (e) are shown as mean ± SEM. Groups marked with different letters (a, b, c, d) are significantly different from each other (p < 0.001). Relative mRNA levels of CXCL12 (f), CXCL11 (g) and CXCR7 (h) in marmoset testes at the age of 16 wpp, 32 wpp, 52 wpp and > 80 wpp were normalized using the housekeeping gene TOP1 (2^ΔΔCt) method. Results are shown as mean ± SEM and significant differences between the groups are marked with *P< 0.05.

**Fig. 2** Immunohistochemical detection of CXCL12 in marmoset testes at the age of 16 wpp (wpp = weeks post partum; a), 32 wpp (c, d), 52 wpp (e, f) and > 80 wpp (g, h). Incubation with non-specific IgG antibody was performed as control and a representative image of a marmoset testis at the age of 16 wpp is shown in (b). During testicular development, the chemokine CXCL12 is exclusively localized in the cytoplasm of Sertoli cells (white arrow). Neither relatively undifferentiated germ cells (gonocytes, arrow head; spermatogonia, black arrow) nor differentiating germ cells showed expression of CXCL12. Scale bars represent 20 µm.
Fig. 3 Immunohistochemical detection of CXCR7 and labelling index of CXCR7-positive cells in marmoset testes at the age of 16 wpp (wpp = weeks post partum). A representative image is showing CXCR7 expression in marmoset testes at the age of 16 wpp (a). The staining revealed that subsets of germ cells express CXCR7 (black arrow), whilst the remainder are negative (arrow head) for CXCR7. Sertoli cells (white arrow) in the seminiferous tubules showed no expression of CXCR7. Corresponding IgG antibody was used as negative control and a representative image is shown in (b). High magnification of CXCR7 stained tissue sections revealed CXCR7-positive and negative gonocytes (c, d), pre-spermatogonia (e, f) and spermatogonia (g, h). Scale bars represent 10 µm. Scores of CXCR7-positive and CXCR7-negative gonocytes (Gon+/Gon−), pre-spermatogonia (pre-SPG+/pre-SPG−) and spermatogonia (SPG+/SPG−) in marmoset testes at the age of 16 wpp are shown in (i). Results are shown as mean ± SEM and significant differences between the groups are marked with *P< 0.05.

Fig. 4 Representative images for expression of MAGE-A4 (green) and CXCR7 (red) in testis sections from marmosets at the age of 16 wpp (a - c). MAGE-A4 expression is seen in the majority of germ cells including gonocytes (d), pre-spermatogonia (g) and spermatogonia (j). Subpopulations of MAGE-A4+CXCR7+ (arrow heads) and/or MAGE-A4+CXCR7− (arrows) gonocytes (d – f), pre-spermatogonia (g – i) as well as spermatogonia (j – l) can be identified. Note that MAGE-A4+CXCR7− pre-spermatogonia are occasionally seen in the seminiferous tubules of marmoset testes at the age of 16 wpp. All sections were counterstained with Hoechst (blue). Dotted lines indicate the basement membrane of the seminiferous tubules. Scale bars represent 10 µm.
Fig. 5 Immunohistochemical detection of CXCR7 in marmoset testes at the age of 32 wpp (wpp = weeks post partum; a, b), 52 wpp (c, d) and > 80 wpp (e, f). In marmoset testes at the age of 32 wpp, 52 wpp and > 80wpp, premeiotic germ cells (black arrow; including A\textsubscript{pale} and A\textsubscript{dark} spermatogonia) located at the basement membrane are labelled for CXCR7 whereas Sertoli cells (white arrow) showed no expression for CXCR7. Scale bars represent 20 µm (a – f). Following enzymatic digestion of adult marmoset testes, haematoxylin-stained single cells showed cell types with different morphology (g) as well as immunofluorescence staining revealed CXCR7-positive cells in this single cell suspension (h). As negative control, incubation with nonspecific IgG antibody was performed and a representative image is shown in (i). Scale bars represent 50 µm (g), 15 µm (h) and 60 µm (i).

Fig. 6 Immunohistochemical detection of CXCR7 in human testes at 2 weeks (a) and 6 months after birth (c) as well as in adult (d) human testes. Corresponding IgG antibody was used as negative control and a representative image of an immature testis (2 weeks) is shown in (b). In immature human testes (a; c), gonocytes (arrow head, no contact with the basement membrane), pre-spermatogonia (white arrows, point contact with the basement membrane) and spermatogonia (black arrows, full contact with the basement membrane) were labelled for CXCR7. In adult human testes (d), premeiotic germ cells (red arrow) located at the basement membrane were labelled for CXCR7. Scale bars represent 20 µm.

Fig. 7 Schematic drawing of CXCR7 (red) and CXCL12 (green) expression in seminiferous tubules of marmoset testes at the ages of 16 wpp (a), 32 wpp (b), 52 wpp (c) and > 80 wpp (wpp = weeks post partum, d). In marmoset testes at the age of 16 wpp, CXCL12 is highly expressed by Sertoli cells whereas the atypical chemokine receptor CXCR7 is expressed by few gonocytes, pre-spermatogonia and spermatogonia (a). In the seminiferous epithelium of...
marmoset testes at the ages of 32 wpp (b), 52 wpp (c) and > 80 wpp (d), CXCL12 is still highly expressed by Sertoli cells whereas CXCR7 is expressed by premeiotic germ cells, including A\textsubscript{pale} and A\textsubscript{dark} spermatogonia. The expression pattern of CXCL12 and CXCR7 indicates a CXCL12/CXCR7 network in primate testes.
Title: Developmental expression patterns of the chemokines CXCL11, CXCL12 and its receptor CXCR7 in common marmoset and human testes

Authors: Birgit Westernströer¹, Daniel Langenstroth¹, Sabine Kliesch², Britta Troppmann¹, Klaus Redmann¹, Joni Macdonald³, Rod Mitchell³, Joachim Wistuba¹, Stefan Schlatt¹, Nina Neuhaus¹

¹ Centre of Reproductive Medicine and Andrology, CeRA, Albert-Schweizer-Campus 1, Building D11, 48149 Münster, Germany
² Department of Clinical Andrology, Centre of Reproductive Medicine and Andrology, CeRA, Albert-Schweitzer-Campus 1, Building D11, 48149 Münster, Germany
³ MRC/University of Edinburgh Centre for Reproductive Health, The Queen´s Medical Research Institute, 47 Little France Crescent, Edinburgh, EH164TJ, Scotland, UK

Corresponding author: Prof. Dr. Stefan Schlatt
Centre of Reproductive Medicine and Andrology
Albert-Schweizer-Campus 1 (D11)
48149 Muenster, Germany
Email: Stefan.Schlatt@ukmuenster.de

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Abstract

The chemokine receptor CXCR7 interacts with the chemokines CXCL11 and CXCL12. During development this ligand receptor system (C-X-C) provokes cell type-specific responses in terms of migration, adhesion or ligand sequestration. It is active in zebrafish and rodents but no data are available for its presence or function in primate testes. Real-time quantitative PCR was performed in the monkeys to detect CXCL11, CXCL12 and CXCR7. At the protein level CXCL12 and CXCR7 were localized in marmoset (*Callitrix jaccus*) testes whereas CXCR7 patterns were determined for various stages of human testes. Morphometry and flow cytometry were applied to quantify CXCR7-positive cells in monkeys. Transcript levels and protein expression of CXCR7 were detectable throughout testicular development. In both species CXCR7 protein expression was restricted to premeiotic germ cells. In immature marmoset testes 69.9 % ± 9% of the total germ cell population were labelled for CXCR7 whereas in the adult 4.7% ± 2.7% were positive for CXCR7. CXCL12 mRNA was detectable in all developmental stages of marmosets. The CXCL12 protein was exclusively localized to Sertoli cells. This pattern of CXCL12/CXCR7 indicates its involvement in regulatory processes that may concert the interaction between undifferentiated germ cells and Sertoli cells.
Introduction:

The (C-X-C-motif) chemokine receptor type 7 (CXCR7) is a deorphanized G-protein-coupled seven-span transmembrane receptor which binds the chemokines CXCL12 (also called stromal-derived factor-1, SDF-1) and CXCL11 (also called interferon-inducible T cell α-chemoattractant, I-TAC) (Balabanian et al. 2005; Burns et al. 2006). Whereas CXCL12 is expressed under homeostatic conditions to modulate immune surveillance and development, the expression of CXCL11 is upregulated upon stimuli and therefore belongs to the inflammatory chemokines. In general, CXCL11 production is stimulated by interferons in a variety of cells, including leukocytes, fibroblasts and endothelial cells (reviewed in: Sánchez-Martin et al. 2013) and therefore supports the massive cell infiltration in several inflamed tissues. The chemokines CXCL12 and CXCL11 bind also to the conventional chemokine receptors CXCR4 and CXCR3, respectively (Balabanian et al. 2005). However, CXCR7 binds CXCL12 and CXCL11 with higher affinity and belongs to the group of atypical chemokine receptors (ACKRs). Interestingly, ACKRs are unable to couple to G-proteins following ligand binding and therefore fail to induce the full spectrum of classical G-protein-coupled chemokine receptor signalling and cellular responses (Comerford et al. 2007; Ulvmar et al. 2011; Cancellieri et al. 2013; Nibbs et al. 2013). Instead, recent studies have demonstrated that CXCR7 can signal through versatile adapter molecules and acts as an endogenous β-arrestin-biased signalling receptor (Rajagopal et al. 2010). In addition, CXCR7 may serve as a co-receptor for CXCR4 and therefore enhances the classical CXCL12-mediated G-protein signalling (Levoye et al. 2009; Luker et al. 2009).

In particular, the action of CXCR7 has been shown to be important for several steps in developing organs (Sánchez-Martin et al. 2013). Interestingly, expression of CXCR7 was detected in various cell types. For instance, expression was found on the cell surface of mesenchymal stem cells (Liu et al. 2010) as well as on renal progenitor cells (Mazzinghi et al. 2008). However, in the primordium of the zebrafish, CXCR7 expression was restricted to...
the somatic environment. More specifically, it has been demonstrated that a CXCL12 gradient in the somatic tissue facilitates the directed migration of CXCR4-positive primordial germ cells (PGCs) towards the future gonad (Boldajipour et al. 2008; Mahabaleshwar et al. 2008; Staton et al. 2011; Mahabaleshwar et al. 2012). In this regard, expression of CXCR7 is of particular importance as it acts as a scavenger for CXCL12 and is therefore responsible for the generation of a CXCL12 gradient (Boldajipour et al. 2008; Mahabaleshwar et al. 2008; Staton et al. 2011; Mahabaleshwar et al. 2012). In addition, the CXCL12/CXCR4 interaction is also of importance for PGC migration in mice, suggesting a conserved mechanism (Ara et al. 2003; Molyneaux et al. 2003).

Moreover, recent studies in juvenile as well as in adult mouse and human testes have revealed that CXCL12 is secreted by Sertoli cells and showed that this chemokine is required for the migration and maintenance of undifferentiated spermatogonia within the stem cell niche (Gilbert et al. 2009; Payne et al. 2010; Kanatsu-Shinohara et al. 2012; Yang et al. 2013). Transcripts of Cxcr7 have been detected in developing mouse testes (Westernströer et al. 2014) as well in testes obtained from adult rats and humans (Eva et al. 1993; McIver et al. 2013). In addition, the cellular localization of Cxcr7 in developing mouse testes has been shown in premeiotic germ cells suggesting that Cxcr7 may be involved in the regulation of the spermatogonial population (Westernströer et al. 2014).

Regarding testicular development and testicular stem cell physiology, the common marmoset monkey is similar to the human (Li et al. 2005; Mitchell et al. 2008; Albert et al. 2010; Albert et al. 2012; Lin et al. 2012; McKinnell et al. 2013) and is therefore an appropriate animal model to study the CXCR7 axis in the developing testis further. Therefore, we used immature and adult human testicular tissues and employed the marmoset monkey (Callitrix jacchus) as a non-human primate animal model in order to elucidate the expression pattern of CXCR7 and its ligands at distinct stages of testicular development.
The primary aim of the present study was to investigate mRNA expression levels of \textit{CXCL12}, \textit{CXCL11} and \textit{CXCR7} in marmoset monkeys during four different phases of testicular development. Based on these results, the second aim was to identify and to characterize the cellular localization of CXCR7 protein expression during testicular development in the marmoset and human testis.

Material and Methods

Animals, collection and processing of testicular tissue

All experimental procedures were performed in compliance with the German Federal Law on the Care and Use of Laboratory Animals (animal license No. 84-02.05.20.12.0.18, LANUV NRW, Germany). Common marmoset monkeys, \textit{Callitrix jacchus}, were maintained at the institutional breeding facilities under standardized conditions. Marmosets were fed with pellets from Altromin (Lage, Germany) with daily supplement of fresh vegetables and fruits. In addition, they had unlimited access to tap water and were kept under 12h light / 12h darkness cycles.

Monkeys (n = 4 / group) were sacrificed under anaesthesia (ketamine/xylazine solution in saline; i.m.; 0.1 ml per 100 gram body weight) at the age of 16, 32, 52 and > 80 weeks post-partum (wpp). Testes were dissected and one half was snap-frozen for subsequent RNA expression analysis, whereas the remaining tissue was fixed in Bouin’s solution for 24 hrs, transferred into 70% EtOH and embedded in paraffin for immunohistochemical analyses.

For flow cytometric analysis, adult marmosets (n = 3) were sacrificed as outlined above. Testes were dissected and transferred into pre-cooled minimum essential medium-alpha (MEM-\(\alpha\), Life Technologies, Gibco, Darmstadt, Germany), followed by enzymatic digestion for flow cytometry.
Human testicular tissues and preparation for histological analyses

Human adult testicular tissues were obtained from patients attending the department of Clinical Andrology due to treatment of testicular tumours. Of such patients testicular tissue which was assessed to be unaffected by the tumour and with complete spermatogenesis was used in this study (Ethics Committee of the Medical Faculty of Muenster and the State Medical Board no. 2006-588-f-S). Status of spermatogenesis was assessed according to Bergmann and Kliesch (2010).

Tissue was fixed in 4% paraformaldehyde (PFA) for 24 hrs, before it was transferred into an increasing ethanol series (30%, 50% and 70%) and embedded in paraffin. For histological evaluation, 3 µm sections were prepared and were stained using periodic acid Schiff’s reagent (PAS staining) followed by haematoxylin counterstaining as previously described (Brinkworth et al. 1995). Based on the histological analysis, testicular tissues (n = 2) with normal spermatogenesis were selected for this study.

In addition, human testicular tissues (age: 2 weeks and 6 months) from the Centre for Reproductive Health at the University of Edinburgh were used. Testes were obtained at autopsy with consent of their legal guardian from boys who died from various causes (excluding reproductive and endocrine abnormalities). Testes were fixed in 10% Neutral Buffered Formalin (NBF) for at least 24 hrs and transferred into 70% EtOH for processing.

Immunofluorescence on testicular tissue

Paraffin was removed using Pro Taqs Clear (Pro Taqs Clear, Quartett Immunodiagnostika & Biotechnologie, Berlin, Germany) and sections were rehydrated in a decreasing ethanol series. After washing with phosphate-buffered saline (PBS), sections were incubated with 5% (w/v) bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 20 min at room
temperature (RT). Primary antibodies against CXCR7 (rabbit polyclonal anti-CXCR7, Abcam, dilution of 1:400, Cambridge, UK) and MAGE-A4 (mouse anti-MAGE-A4, provided by Prof. G. C. Spagnoli from the University Hospital of Basel Switzerland, dilution of 1:20) were applied to the sections and sections were incubated in a humid chamber at 4°C for 1 hr. Following two washing steps in PBS, the appropriate Alexa fluor 488-linked or 546-linked secondary antibodies, diluted in TBS/5% BSA, were applied for 45 min at RT in the dark. Incubation with non-specific immunoglobulin G (rabbit IgG and mouse IgG, dilution of 1:1000) was used as negative control. Cells were counterstained with Hoechst (Sigma-Aldrich, Steinheim, Germany).

**Immunohistochemistry on testicular tissue**

Sections were dewaxed and rehydrated as described above. After rinsing with distilled water and TBS, non-specific peroxidases were blocked with 3% (v/v) H$_2$O$_2$ for 15 min at RT. In order to block non-specific binding sites, sections were incubated with 5% (w/v) bovine serum albumin (BSA) for 20 min at RT. Subsequently, primary antibodies against CXCR7 (rabbit polyclonal anti-CXCR7, Abcam, dilution of 1:400, Cambridge, UK) and CXCL12 (mouse monoclonal anti-CXCL12, R&D system, dilution of 1:50, Wiesbaden, Germany) were applied and sections were incubated overnight in a humid chamber at 4°C. Incubation with corresponding non-specific immunoglobulin G (rabbit IgG or mouse IgG, respectively) antibodies served as negative control. After three washing times with TBS, sections were incubated with biotin conjugated secondary antibodies and subsequently labeled with a streptavidin-conjugated horseradish peroxidase (Sigma-Aldrich, Steinheim, Germany). Staining was visualized using 3,3’-diaminobenzidine as chromogen (Sigma-Aldrich, Steinheim, Germany), and haematoxylin as counterstain.
Morphometric evaluation

For morphometric evaluation of marmoset testicular tissues, 4-µm sections were taken at 40-µm intervals. In tissue sections from infantile marmosets, a total of 100 seminiferous tubules per animal (n = 3) were analyzed to determine labeling indices of CXCR7-positive germ cells. In accordance with previous publications germ cell types were categorized into the following three groups, cells with no observable contact with the basement membrane were classified as gonocytes, those with some point of contact as pre-spermatogonia and those with full contact with the basement membrane as spermatogonia (Sharpe et al. 2003b). Additionally, the percentages of CXCR7-positive and negative gonocytes, pre-spermatogonia and spermatogonia were determined.

RNA isolation and relative gene expression analysis

RNA extraction was performed using the miRNeasy Kit (Quiagen, Hilden, Germany) and genomic DNA was removed by DNase treatment. cDNA was generated with the iScript cDNA Synthesis Kit (Bio-Rad, Munich, Germany) using 200 ng of total RNA. Specific primers were designed using Primer Express® 3.0 Software (Life Technologies, Darmstadt, Germany). Optimal primer concentrations, primer specificity and PCR efficiency were evaluated following the Power SYBR® Green PCR User Guide (Life Technologies, Darmstadt, Germany). Primer sequences are summarized in Table 1. For quantitative real-time PCR (qRT-PCR) analyses, cDNA was diluted 1:10 and 1.5 µl were used for each 15 µl PCR reaction with Power SYBR® Green Mastermix (Life Technologies, Darmstadt, Germany). The PCR programme consisted of initial steps of activation and denaturation which were run once for 10 min at 95°C, followed by 40 cycles of denaturation (15 sec at 95°C), annealing and elongation (1 min at 60°C). qRT-PCRs were run on the StepOnePlus™ (Life Technologies, Darmstadt, Germany) and were subsequently analysed using the
StepOne™ software 2.2 (Life Technologies, Darmstadt, Germany). To calculate relative expression levels of CXCR7 (n = 4 / group), CXCL12 (n = 4 / group) and CXCL11 (n = 4 / group) in marmoset testes, the 2^(-ΔCT) method (Livak et al. 2001) was applied, using TOP1 as reference gene. Each sample was measured in duplicate.

Enzymatic digestion and flow cytometry of testicular cells

Single-cell suspension of testicular cells was prepared by sequential enzymatic digestion. The seminiferous tubules were digested in MEM-α with 1 mg/mL collagenase I (Sigma Aldrich, Steinheim, Germany) in a water bath at 37°C for 40 min. The digestion was stopped by adding MEM-α containing 10% fetal bovine serum (FBS, Life Technologies, PA A, Darmstadt, Germany). Afterwards, the seminiferous tubular fragments were isolated from interstitial cells by sedimentation at unit gravity and the seminiferous tubular fragments were further digested with 1 mg/mL collagenase I, 2.2 mg/mL DNase I (Sigma Aldrich, Steinheim, Germany) and 0.5 mg/mL hyaluronidase (Sigma Aldrich, Steinheim, Germany) in MEM-α until a single-cell suspension was achieved. Finally, the single-cell suspension was centrifuged (1500 rpm, 4 min), the supernatant was removed and enzymatic digestion was stopped with FBS.

For haematoxylin staining, testicular single cells were fixed on poly L-lysine coverslips with 4% PFA for 15 min. After rinsing with PBS, cells were counterstained with haematoxylin at RT for 1 min and afterwards analyzed.

For flow cytometric analysis, single-cell suspensions were filtered through a 30 µm mesh and washed with 5% (w/v) goat serum. Subsequently, cells were collected by centrifugation (1500 rpm, 5 min), the supernatant was removed and cells were incubated with the primary antibody against CXCR7 (rabbit polyclonal anti-CXCR7, Abcam, dilution of 1:200, Cambridge, UK) on ice for 30 min. Incubation with the corresponding non-specific
immunoglobulin G (rabbit IgG, dilution of 1:2000) served as negative control. Following two washing steps in 5% (w/v) goat serum, cells were incubated on ice with an Alexa fluor 488-linked secondary antibody (goat anti-rabbit Alexa fluor 488, Life Technologies, Darmstadt, Germany, diluted in 5% goat serum for 30 min. Finally, stained cells were analysed using a BD FACSaria II (Becton Dickinson).

Immunofluorescence staining of testicular single cells

Fixed testicular single cells on poly-L-lysine coverslips were rinsed with PBS. Cells were then incubated with 5% (w/v) BSA at RT for 30 min. Subsequently, primary antibodies against CXCR7 (rabbit polyclonal anti-CXCR7, Abcam, dilution of 1:400, Cambridge, UK) were applied and cells were incubated in a humid chamber at 4 °C for 1 hr. Following two washing steps in PBS, the appropriate Alexa fluor 546-linked secondary antibody, diluted in TBS/5% BSA, was applied for 30 min at RT in the dark. Incubation with non-specific immunoglobulin G (rabbit IgG, dilution of 1:2000) was performed as negative control. Cells were counterstained with Hoechst (Sigma-Aldrich, Steinheim, Germany).

Histological sample evaluations and image acquisition

Samples were analyzed using an Olympus BX61 microscope (Melville, NY, USA) with an attached Retiga 400R camera (QImaging, Burnaby, BC, Canada). All images were acquired digitally using cellSens imaging software (Olympus, Münster, Germany) and were processed using Adobe Photoshop CS2 9.0 (Adobe Systems, CA, USA).
**Statistical analysis**

Statistical analyses were performed using Graph Pad Prism 5 (Graph Pad Software, USA). For analysis of qRT-PCR results the non-parametric Kruskal-Wallis test was performed followed by the Dunn’s multiple comparison test. For morphometric analyses the Student’s t-test was applied. For these two tests, significant differences between the groups are marked with *P<0.05.

Significant differences between the testis weights were determined using the “One way analysis of variance” followed by the “Tukey’s multiple comparison test”. Groups marked with different letters (a, b, c, d) are significantly different from each other (P<0.001).
Results

Testicular development, testis growth and expression pattern of CXCL12, CXCL11 and CXCR7 in postnatal marmoset testes.

Testicular tissues from four phases of testicular development (16 wpp, 32 wpp, 52 wpp and > 80 wpp) were selected to characterize the expression pattern of CXCR7, CXCL12 and CXCL11 in the marmoset monkey.

Histological examination of testicular tissues of marmoset monkeys at the age of 16 wpp (Fig. 1a) revealed the presence of gonocytes, pre-spermatogonia and spermatogonia. At the age of 32 wpp, differentiated germ cells up to spermatocytes were detected in seminiferous tubules of marmoset monkeys (Fig. 1b). At the age of 52 wpp (Fig. 1c), round as well as elongated spermatids were observed in the seminiferous tubules. Finally, spermatogenesis was completed in the seminiferous tubules of marmoset monkeys at the age of > 80 wpp (Fig. 1d). In these age groups, testis weights (Fig. 1e) increased significantly from 36.4 ± 6.3 mg (16 wpp) via 181.9 ± 35.7 mg (32 wpp) and 376.7 ± 72.0 mg (52 wpp) to 481.9 ± 36.5 mg (> 80 wpp).

Relative expression levels of CXCR7 (n = 4 / group), CXCL12 (n = 4 / group) and CXCL11 (n = 4 / group) during postnatal testis development in the marmoset were determined using qRT-PCR analysis. Expression of CXCL12 was high in marmoset testes at the age of 16 wpp (0.214445 ± 0.112887) and then decreased significantly (0.088170 ± 0.012248; 2.4-fold; 52 wpp) during testicular development (Fig.1f). Compared to CXCL12, expression of CXCL11 was 56-fold (16 wpp), 76-fold (32 wpp), 21-fold (52 wpp) and 23-fold lower (> 80 wpp) and did not change significantly during postnatal testis development (Fig. 1g). Expression of the receptor CXCR7 (Fig. 1h) was high in marmoset testes at the age of 16 wpp (0.368448 ± 0.094921) and decreased significantly (0.092540 ± 0.013916; 4-fold; > 80 wpp) during postnatal development.
CXCL12 and CXCR7 protein expression in postnatal marmoset testes

Immunohistochemistry was performed to determine the expression of the homeostatic chemokine CXCL12 and the corresponding receptor CXCR7 during postnatal testis development in marmoset monkeys.

In the testes of marmoset monkeys at the age of 16 wpp (Fig. 2a), 32 wpp (Fig. 2c, d), 52 wpp (Fig. 2e, f) and > 80 wpp (Fig. 2g, h), CXCL12 was exclusively localized in the cytoplasm of Sertoli cells. In contrast, expression of CXCR7 was restricted to the germ cell population. More specifically, in marmoset testes at the age of 16 wpp the expression of CXCR7 was found in a subset of gonocytes (Fig. 3c), pre-spermatogonia (Fig. 3e) and spermatogonia (Fig. 3g). Furthermore, morphometric analyses revealed that the CXCR7 labelling index of the entire germ cell population was 69.9 ± 9.0% (Fig. 3i), which comprised CXCR7-positive gonocytes (Fig. 3c), pre-spermatogonia (Fig. 3f) and spermatogonia (Fig. 3h) with the indices of 36.8 ± 13.7%, 18.0 ± 4.1% and 15.1 ± 9.8%, respectively (Fig. 3i).

Double immunofluorescence stainings (Fig. 4a-l) revealed subpopulations of MAGE-A4+/CXCR7+ and MAGE-A4+/CXCR7- gonocytes (Fig. 4d, e, f), pre-spermatogonia (Fig. 4g, h, i) and spermatogonia (Fig. 4j, k, l). However, subsets of MAGE-A4+/CXCR7- pre-spermatogonia (Fig. 4g, h, i) were only rarely observed in tubules of marmoset testes at the age of 16 wpp.

During postnatal development CXCR7 expression persisted in premeitoic germ cells located at the basement membrane (Fig. 5a - f). CXCR7-positive A_pale (Fig. 5b, d) and A_dark (Fig. 5f) spermatogonia were identified by morphological evaluation in marmoset monkeys at the age of 32 wpp, 52 wpp and > 80 wpp.

Furthermore, adult marmoset testes (n = 3) were enzymatically digested and isolated single cells were subjected to haematoxylin staining which showed cell types with different sizes and morphology (Fig. 5g). Subsequent, flow cytometric assessment revealed expression of
CXCR7 on the cell surface of 4.7 ± 2.7% of cells in the single cell suspension obtained from adult marmoset testes (Table S2) and CXCR7 protein expression was confirmed by immunofluorescence staining of cells fixed on poly L-lysine slides (Fig. 5h). The qualitative observations of CXCR7-positive cells following immunofluorescence staining were consistent with the data obtained by flow cytometry.

**CXCR7 protein profiles in postnatal human testes**

A panel of rare postnatal human testis samples (2 weeks, 6 months and adult) was immunohistochemically stained and revealed that a subset of gonocytes, pre-spermatogonia as well as spermatogonia was positive for CXCR7 in immature human testes (Fig. 6a-c). Moreover, the expression of CXCR7 in adult human testes (Fig. 6d) was found in premeiotic germ cells at the basement membrane.

**Discussion**

We have illustrated a schematic drawing of the localization patterns of CXCL12 and CXCR7 which are pointing to a crucial role of this chemokine/receptor pair in the developing primate testis (Fig. 7). Interestingly, transcript levels of CXCL12 were constitutively expressed and immunohistochemical analyses further demonstrated protein expression of CXCL12 in the cytoplasm of Sertoli cells at all stages of postnatal testicular development which indicated a non-maturation-dependent expression of the homeostatic chemokine CXCL12 in the developing marmoset testis. After birth, the highest number of Sertoli cells per testis is established at around 4 month of age. Afterwards, the Sertoli cell population decreases and after 8 month of age Sertoli cells become morphologically mature (e.g. establishment of tight-junctions between adjacent Sertoli cells, formation of lumen in the seminiferous tubules) in...
marmoset testes (Sharpe et al. 2003a; Li et al. 2005). We therefore assume this phenomenon to explain the significantly decreased transcript levels of CXCL12 in the developing marmoset testis. The Sertoli cell-specific expression pattern of CXCL12 described in this study is in line with previous mouse (Payne et al. 2010; Yang et al. 2013, Westernströer et al. 2014) and human data (Gilbert et al. 2009). Here we describe for marmosets a decline of CXCL12 from week 16-52 (prepuberty to postpuberty). This mRNA pattern is different from that previously observed in mice. The mRNA levels of Cxcl12 in developing mouse testes remained constant from birth until day 21 and then increased until adulthood (Westernströer et al. 2014). The two methods for quantification of mRNA are different (mouse: luciferase based normalization; marmoset: reference gene based normalization) and the reported levels can therefore not be easily compared. However in mice and marmosets transcripts for CXCL12 are detectable and appear to be modified throughout pubertal development. Current results therefore suggest that the action of CXCL12 is evolutionarily conserved. Functions of CXCL12 have been described to be organ or tissue specific. This includes a role for cell proliferation or survival (Kortesidis et al. 2005; Burns et al. 2006; Mazzinghi et al. 2008), differentiation (Lazarini et al. 2003), adhesion (Burns et al. 2006; Mazzinghi et al. 2008) and the regulation of cell migration (Bhakta et al. 2006; Mazzinghi et al. 2008). Regarding germ cells, CXCL12 has been shown to be secreted by the somatic environment thereby facilitating the colonization of the gonads by PGCs during mouse embryonic development (Doitsidou et al. 2002; Ara et al. 2003; Molyneaux et al. 2003; Dambly-Chaudiere et al. 2007; Boldajipour et al. 2008). Furthermore, recent studies in neonatal and adult mice revealed that CXCL12 is required for maintenance of spermatogonial stem cells (SSCs) within their niches as well as for the homing and colonization process of SSCs into their niches following germ cell transplantation assays (Payne et al. 2010; Kanatsu-Shinohara et al. 2012; Yang et al. 2013). Moreover, we previously reported significantly increased transcript levels of Cxcl12 following germ cell
depletion in mice and suggested that Cxcl12 may stimulate the spermatogenic repopulation of the seminiferous tubules during testicular recovery (Westernströer et al. 2014).

Whereas inflammatory chemokines (e.g. the C-X-C chemokine CXCL11) are mainly involved in the recruitment of leukocytes to inflamed tissue sites, homeostatic chemokines (e.g. the C-X-C chemokine CXCL12) are constitutively expressed in organs and have important functions in stem cell homing, tissue homeostasis and repair. Indications for testicular inflammation (e.g. massive cell infiltration by neutrophils, lymphocytes, macrophages and mast cells in the interstitium) (Guazzone et al. 2009) were not seen in the analyzed testes. Moreover, in contrast to CXCL12, only low transcript levels of CXCL11 were detected in developing marmoset testes which confirmed that inflammatory C-X-C chemokines, especially CXCL11 as a secondary ligand for CXCR7, is likely not of functional relevance for healthy marmoset testes.

Transcript levels of CXCR7 were also constitutively expressed and immunohistochemical analyses further demonstrated that the atypical chemokine receptor CXCR7 is exclusively expressed by premeiotic germ cells (gonocytes, pre-spermatogonia, spermatogonia including A_pale and A_dark spermatogonia) throughout various phases of postnatal testicular development in the marmoset and the human testis. In agreement with these data we found that Cxcr7 protein can be detected in gonocytes as well as in Sall4-positive undifferentiated spermatogonia of the developing mouse testis (Westernströer et al. 2014). Additionally, Cxcr7 was also expressed on differentiated germ cells, spermatids and interstitial cells. These results reveal an evolutionarily conserved expression pattern of CXCR7 in premeiotic germ cells in the mouse and both analysed primate species (Westernströer et al. 2014). The morphometric evaluation in immature marmoset testes has shown additionally that approximately 80% of migrating germ cells (gonocytes and pre-spermatogonia) express CXCR7. In general, recent studies in marmoset monkeys indicated that the migration of gonocytes most likely begins postnatally between birth and 4 months of age (Rune et al. 2016).
Interestingly, several MAGE-A4+/CXCR7+ gonocytes were observed, whereas subsets of MAGE-A4+/CXCR7+ pre-spermatogonia were only rarely seen in seminiferous tubules, suggesting that CXCR7 may be involved in migration and/or adhesion of germ cells in immature marmoset testes. In general, migration of centrally located germ cells into the basal part of the seminiferous epithelium is a cellular process critical for germ cell survival and establishment of A<sub>dark</sub> and A<sub>pale</sub> spermatogonia which represent the reserve and active SSC pool in adulthood in marmoset and human testis (McGuinness and Orth, 1992; Orth et al. 2000). Moreover, our morphometric evaluation in immature marmoset testes has shown that around 50% of the spermatogonia at the basement membrane express CXCR7. Furthermore, we demonstrated that A<sub>dark</sub> and A<sub>pale</sub> spermatogonia express CXCR7 in marmoset testes at the age of 32 wpp, 52 wpp and > 80 wpp. Therefore, we assume different roles for the atypical chemokine receptor CXCR7 in the developing marmoset testes. Tissue- and cell type-specific functions of CXCR7 have been described and depend on the cell type and tissue. This includes a role for cell survival or adhesion as described for example for in the breast cancer cell line MCF-7 (Burns et al. 2006) as well as in renal multipotent progenitor cells of the human kidney (Mazzinghi et al. 2008).

In addition, using HEK-293 and MDA-MB-231 cells, it has been demonstrated that receptor heterodimerization between CXCR4 and CXCR7 may enhance CXCL12-induced cell migration (Levoye et al. 2009; Decaillot et al. 2011). Regarding the localization of CXCR4 in developing mammalian testes, different expression patterns have been shown for mouse and human (Gilbert et al. 2009; Yoon et al. 2009; Kanatsu-Shinohara et al. 2012; Yang et al. 2013, McIver et al. 2013). Whilst expression of CXCR4 was found in gonocytes, undifferentiated spermatogonia, Sertoli cells and interstitial cells of neonatal and adult mouse testes (Yoon et al. 2009; Kanatsu-Shinohara et al. 2012; Yang et al. 2013), CXCR4 protein was detected throughout the seminiferous tubules of adult human testes (Gilbert et al. 2009).
So far, the expression profile and the role of CXCR4 in developing marmoset testes remain unknown. Nevertheless, receptor heterodimerization between CXCR4 and CXCR7 throughout testicular development in primate testes cannot be excluded but needs to be investigated in further studies.

In conclusion, we show that CXCL12/CXCR7 network is present in primate testes. The localization pattern suggests its involvement during migration, survival or adhesion in premeiotic germ cells.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figures:

**Fig. 1** Marmoset monkey testes after birth - age-related changes in histology, weight and transcript levels. Micrographs showing PAS stained tissue sections of marmoset testes at the age of 16 wpp (wpp = weeks post partum; a), 32 wpp (b), 52 wpp (c) and > 80 wpp (d). Gonocytes (arrowhead), pre-spermatogonia (white arrow) and spermatogonia (black arrow) are the predominant germ cells in marmoset testes at the age of 16 wpp (a). Meiotic cells (primary spermatocytes) were present in the seminiferous tubules of marmosets at the age of 32 wpp (b), whilst germ cells up to the stage of elongated spermatids were detected in the seminiferous epithelium of marmoset monkeys at the age of 52 wpp (c). Finally, spermatogenesis was complete in all marmosets at the age of > 80 wpp (d). Scale bars represent 50 µm. Changes in testis weight (e) are shown as mean ± SEM. Groups marked with different letters (a, b, c, d) are significantly different from each other (p < 0.001). Relative mRNA levels of CXCL12 (f), CXCL11 (g) and CXCR7 (h) in marmoset testes at the age of 16 wpp, 32 wpp, 52 wpp and > 80 wpp were normalized using the housekeeping gene TOP1 (2^(-ΔΔCt)) method. Results are shown as mean ± SEM and significant differences between the groups are marked with *P< 0.05.

**Fig. 2** Immunohistochemical detection of CXCL12 in marmoset testes at the age of 16 wpp (wpp = weeks post partum; a), 32 wpp (c, d), 52 wpp (e, f) and > 80 wpp (g, h). Incubation with non-specific IgG antibody was performed as control and a representative image of a marmoset testis at the age of 16 wpp is shown in (b). During testicular development, the chemokine CXCL12 is exclusively localized in the cytoplasm of Sertoli cells (white arrow). Neither relatively undifferentiated germ cells (gonocytes, arrow head; spermatogonia, black arrow) nor differentiating germ cells showed expression of CXCL12. Scale bars represent 20 µm.
**Fig. 3** Immunohistochemical detection of CXCR7 and labelling index of CXCR7-positive cells in marmoset testes at the age of 16 wpp (wpp = weeks post partum). A representative image is showing CXCR7 expression in marmoset testes at the age of 16 wpp (a). The staining revealed that subsets of germ cells express CXCR7 (black arrow), whilst the remainder are negative (arrow head) for CXCR7. Sertoli cells (white arrow) in the seminiferous tubules showed no expression of CXCR7. Corresponding IgG antibody was used as negative control and a representative image is shown in (b). High magnification of CXCR7 stained tissue sections revealed CXCR7-positive and negative gonocytes (c, d), pre-spermatogonia (e, f) and spermatogonia (g, h). Scale bars represent 10 µm. Scores of CXCR7-positive and CXCR7-negative gonocytes (Gon+/Gon–), pre-spermatogonia (pre-SPG+/pre-SPG–) and spermatogonia (SPG+/SPG–) in marmoset testes at the age of 16 wpp are shown in (i). Results are shown as mean ± SEM and significant differences between the groups are marked with *P< 0.05.

**Fig. 4** Representative images for expression of MAGE-A4 (green) and CXCR7 (red) in testis sections from marmosets at the age of 16 wpp (a - c). MAGE-A4 expression is seen in the majority of germ cells including gonocytes (d), pre-spermatogonia (g) and spermatogonia (j). Subpopulations of MAGE-A4+/CXCR7– (arrow heads) and/or MAGE-A4+/CXCR7+ (arrows) gonocytes (d – f), pre-spermatogonia (g – i) as well as spermatogonia (j – l) can be identified. Note that MAGE-A4+/CXCR7+ pre-spermatogonia are occasionally seen in the seminiferous tubules of marmoset testes at the age of 16 wpp. All sections were counterstained with Hoechst (blue). Dotted lines indicate the basement membrane of the seminiferous tubules. Scale bars represent 10 µm.
Fig. 5 Immunohistochemical detection of CXCR7 in marmoset testes at the age of 32 wpp (wpp = weeks post partum; a, b), 52 wpp (c, d) and > 80 wpp (e, f). In marmoset testes at the age of 32 wpp, 52 wpp and > 80 wpp, premeiotic germ cells (black arrow; including A<sub>pale</sub> and A<sub>dark</sub> spermatogonia) located at the basement membrane are labelled for CXCR7 whereas Sertoli cells (white arrow) showed no expression for CXCR7. Scale bars represent 20 µm (a – f). Following enzymatic digestion of adult marmoset testes, haematoxylin-stained single cells showed cell types with different morphology (g) as well as immunofluorescence staining revealed CXCR7-positive cells in this single cell suspension (h). As negative control, incubation with nonspecific IgG antibody was performed and a representative image is shown in (i). Scale bars represent 50 µm (g), 15 µm (h) and 60 µm (i).

Fig. 6 Immunohistochemical detection of CXCR7 in human testes at 2 weeks (a) and 6 months after birth (c) as well as in adult (d) human testes. Corresponding IgG antibody was used as negative control and a representative image of an immature testis (2 weeks) is shown in (b). In immature human testes (a; c), gonocytes (arrow head, no contact with the basement membrane), pre-spermatogonia (white arrows, point contact with the basement membrane) and spermatogonia (black arrows, full contact with the basement membrane) were labelled for CXCR7. In adult human testes (d), premeiotic germ cells (red arrow) located at the basement membrane were labelled for CXCR7. Scale bars represent 20 µm.

Fig. 7 Schematic drawing of CXCR7 (red) and CXCL12 (green) expression in seminiferous tubules of marmoset testes at the ages of 16 wpp (a), 32 wpp (b), 52 wpp (c) and > 80 wpp (wpp = weeks post partum, d). In marmoset testes at the age of 16 wpp, CXCL12 is highly expressed by Sertoli cells whereas the atypical chemokine receptor CXCR7 is expressed by few gonocytes, pre-spermatogonia and spermatogonia (a). In the seminiferous epithelium of 28
marmoset testes at the ages of 32 wpp (b), 52 wpp (c) and > 80 wpp (d), CXCL12 is still highly expressed by Sertoli cells whereas CXCR7 is expressed by premeiotic germ cells, including $A_{\text{pale}}$ and $A_{\text{dark}}$ spermatogonia. The expression pattern of CXCL12 and CXCR7 indicates a CXCL12/CXCR7 network in primate testes.
219x220mm (300 x 300 DPI)
325x228mm (300 x 300 DPI)
Supplemental Table 1: Westernströer et al.

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*cal = Callitrix jacchus*
### Flow cytometric assessment of CXCR7-positive cells in adult marmoset testes (n = 3)

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<tr>
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SD = Standard derivation