The C-X-C signalling system in the rodent vs primate testis: impact on germ cell niche interaction

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Abstract

In zebrafish, action of the chemokine Cxcl12 is mediated through its G-protein-coupled seven-transmembrane domain receptor Cxcr4 and the atypical receptor Cxcr7. Employing this animal model, it was revealed that this Cxcl12 signalling system plays a crucial role for directed migration of primordial germ cells (PGC) during early testicular development. Importantly, subsequent studies indicated that this regulatory mechanism is evolutionarily conserved also in mice. What is more, the functional role of the CXCL12 system does not seem to be limited to early phases of testicular development. Data from mouse studies rather demonstrate that CXCL12 and its receptors are also involved in the homing process of gonocytes into their niches at the basal membrane of the seminiferous tubules. Intriguingly, even the spermatogonial stem cells (SSCs) present in the adult mouse testis appear to maintain the ability to migrate towards a CXCL12 gradient as demonstrated by functional *in vitro* migration assays and *in vivo* germ cell transplantation assays. These findings not only indicate a role of the CXCL12 system throughout male germ cell development in mice but also suggest that this system may be evolutionarily conserved. In this review, we take into account the available literature focusing on the localization patterns of the CXCL12 system not only in rodents but also in primates, including the human. Based on these data, we discuss whether the CXCL12 system is also conserved between rodents and primates and discuss the known and potential functional consequences.

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Introduction

The ability of cell migration is a key feature of early germ cells. Following specification at extraembryonic locations, germ cells need to move to the target sites of the developing gonads. Employing zebrafish as animal model, the interaction of the chemokine Cxcl12 with its receptors Cxcr4 and Cxcr7 was shown to be decisive for the directionality of primordial germ cell (PGC) migration (Richardson & Lehmann 2010). Specifically, isoform Cxcl12a is expressed by somatic cells and acts through the G protein-coupled receptor Cxcr4b localized to the cell surface of PGCs and induces migration (Doitsidou *et al.* 2002). In addition, Cxcl12a also acts through the alternative receptor Cxcr7, which is expressed throughout somatic tissues. However, in contrast to Cxcr4 binding to Cxcr7 rather results in internalization of the chemokine and as a consequence of this in formation of a Cxcl12a gradient (Doitsidou *et al.* 2002, Knaut *et al.* 2003, Boldajipour *et al.* 2008, Mahabaleshwar *et al.* 2008, Raz & Mahabaleshwar 2009, Zohni *et al.* 2012, Bussmann & Raz 2015). First insights regarding these early processes of PGC migration have been gained employing the zebrafish, exploiting the fast extrauterine development of embryos as well as

the transparency, which facilitates *in vivo* analyses of selected strains (Doitsidou *et al.* 2002, Bussmann & Raz 2015). Later studies revealed that the CXCL12/CXCR4 interaction is also crucial for directed PGC migration in the mouse (Molyneaux *et al.* 2003). For unveiling the role of this chemokine system for subsequent stages of germ cell development in mice, it was a clear advantage that protocols for the isolation of spermatogonial stem cells (SSCs) as well as the germ cell transplantation assay are established, facilitating functional migration studies *in vitro* and *in vivo*. Following PGC migration, the next stage requiring germ cell migration is the migration of gonocytes (also referred to as prospermatogonia) from the centre of the seminiferous tubules towards the basal lamina and into their stem cell niches. In mice, this process again is mediated by the interaction of CXCL12 and CXCR4 (Payne *et al.* 2010, Gallagher *et al.* 2013), indicating that this regulatory mechanism remains active beyond the phase of germ cell migration towards the gonads. In the adult testis, the ability of germ cell migration may not be obviously required in tissue homeostasis. Yet, the repopulation of seminiferous tubules following gonadotoxic insult and depletion of spermatogonia involves the migration of remaining spermatogonia prior to homing into vacated niches. It

can be postulated that the CXCL12 system remains a constitutive part of the germ cell niche throughout all stages of germ cell development or is reactivated in these cases of gonadotoxic insult. The purpose of this review is to discuss the functional roles of the CXCL12 system throughout germ cell development focusing on data obtained in rodents and primates.

General characteristics and function of CXCL12 and its receptors CXCR4 and CXCR7

Chemotactic cytokines, so-called chemokines, are small proteins (8–14 kDa) that regulate the migration of cells. Their action is mediated via binding to distinct G-protein-coupled seven-transmembrane (7TM) domain receptors (de Vries et al. 2006, Zlotnik et al. 2006). Chemokines are grouped into inflammatory and homeostatic chemokines. Homeostatic chemokines are constitutively expressed and involved in various developmental processes. Amongst the homeostatic chemokines the CXCL12 (chemokine (C-X-C motif) ligand 12)/CXCR4 (chemokine (C-X-C motif) receptor 4) system is essential for the development of the heart, brain and large vessels during mouse embryo development (Ma et al. 1998, Zou et al. 1998). In addition to CXCR4, CXCL12 can also bind to the atypical chemokine receptor CXCR7 (Balabanian et al. 2005, Burns et al. 2006). Binding affinity of CXCL12 to this atypical receptor is even 10× higher compared to CXCR4 (Burns et al. 2006). Structurally, receptors belonging to the group of atypical chemokine receptors also have the classical 7-transmembrane structure that is typical of G protein-coupled chemokine receptors, however, due to a modified motive, binding of the chemokine does not induce classical signalling response. Nonetheless, significance of CXCR7 function for embryo development was demonstrated by the lethal phenotype of CXCR7-knockout mice. Importantly, though, these knockout mice die due to cardiovascular defects, a phenotype different from defects observed in CXCL12- or CXCR4-knockout mice (Sierra et al. 2007, Gerrits et al. 2008).

Functional role of the CXCL12 system for the migration of PGCs and colonization of the gonads

Primordial germ cells (PGCs) are specified early during mammalian embryonic development. They can first be

identified at the embryonic yolk sac from where they migrate via the hindgut to the gonadal ridge (Aeckerle et al. 2012, 2015, Saitou & Yamaji 2012, de Felici 2013, Tang et al. 2015, 2016). PGCs increase in number during the process of migration and maintain their proliferative activity during subsequent colonization of the genital ridges. The general steps of germ cell specification and migration but not the timing of these processes are comparable in mammals (Table 1).

Insights regarding the role of the Cxcl12 system for the process of germ cell migration have been gained in zebrafish. Benefit of this animal model was that early developmental stages are almost transparent enabling the identification and tracking of PGCs in life animals. In this animal model, specification of germ cells occurs at four different positions and germ cells subsequently migrate mediated through the Cxcl12/Cxcr4/Cxcr7 system to the sites of the developing genital ridge (Doitsidou et al. 2002, Raz 2004). Loss of the chemokine or its receptor in zebrafish resulted in PGCs showing a random rather than a directed migration pattern (Raz 2004, Richardson & Lehmann 2010, Wei & Liu 2014). High conservation of Cxcl12 amino acid sequence between zebrafish and mice rendered a conserved function of this chemokine likely (Doitsidou et al. 2002).

To determine the functional role of CXCL12/CXCR4 interaction during the process of mouse PGC migration, CXCR4-mutant mice were compared to age-matched controls. Until day E9.5 germ cell development progressed normally demonstrating that the interaction of CXCL12/CXCR4 is not required for germ cell specification or for initial migration (Molyneaux et al. 2003). Of note, this is different from findings in the zebrafish as mutations as well as oligo-mediated loss of functions resulted in an impaired migration from the start (Doitsidou et al. 2002, Knaut et al. 2003). In the mouse though, CXCL12 promoted directed migration of PGCs and in particular the homing process of PGCs into the gonads.

Whether the CXCL12 system is also directing human PGC migration remains hitherto unknown, but data gained from cell line experiments, suggest that this may indeed be the case. Two studies employed TCam-2 cells, a human testicular germ cell tumour-derived cell line (Gilbert et al. 2010, Mclver et al. 2013), which share key characteristics with PGCs also regarding their gene

Table 1 Chronology of important events during prenatal development of mice, marmosets and humans.

Event	Age			References
	Mouse	Marmoset	Human	
PGC commitment and specification (epiblast)	E6.25–6.5	Unknown	E12–16	Saitou & Yanaji (2012), Tang et al. (2015)
Identification of PGCs at the yolk sac	E7.5–8	E50	E21–28	Saitou & Yanaji (2012), De Felici (2013), Tang et al. (2015), Aeckerle et al. (2015)
Localization of PGCs at the genital ridge	E10.5–11.5	E65–75	E35–42	Saitou & Yanaji (2012), De Felici (2013), Tang et al. (2015), Aeckerle et al. (2015)
Differentiation of gonadal anlage into testes	E12	E75	E42	Li et al. (2005), Saitou & Yanaji (2012), De Felici (2013), Tang et al. (2015)

expression profile (Irie *et al.* 2015). Particularly, TCam-2 cells show expression of CXCR4 as well as CXCR7 (Gilbert *et al.* 2010). Expression of these cell surface receptors was of functional relevance as demonstrated by migration assays using a Boyden chamber approach with medium supplemented with CXCL12 (Mclver: 50, 100, 200 ng/mL; Gilbert: 100 ng/mL) in the lower chamber. Compared to a no-gradient control the ratio of migrating cells increased significantly in response to CXCL12. Interestingly, adding the CXCR4-specific antagonist AMD3100 (Mclver: 25 nM; Gilbert: 2.5 ng/mL) to the lower chamber or applying a CXCR4-specific knockdown reduced the number of migrating cells significantly in comparison to the no-gradient control despite the presence of a CXCL12 gradient (Gilbert *et al.* 2010, Mclver *et al.* 2013). Conclusively, the influence of the CXCL12/CXCR4 axis on the migration of TCam-2 cells provides a first indication that the chemokine and its receptor may also play a role in migration of human PGCs.

The spermatogonial stem cell niche and the role of the CXCL12 system

Following PGC migration into the developing gonads, germ cells colonize the genital ridges. Early male germ cells, which are now located within the seminiferous tubules of the gonad are referred to as gonocytes. In mice, gonocytes enter a mitotically quiescent phase and shortly after birth resume proliferation and migrate from the center of the cords to the basement membrane and give rise to the spermatogonial population including the spermatogonial stem cells (SSC) (Nagano *et al.* 2000, Drumond *et al.* 2011, Saitou & Yamaji 2012). In primates, fetal germ cells proliferate throughout gestation and germ cell proliferation continues until the first weeks after birth (Honecker *et al.* 2004, Mitchell *et al.* 2008). Comparable to rodents, gonocytes in primate testicular tissues migrate to the basement membrane and differentiate into spermatogonia after birth. However, this process spans a longer period in humans as well as in marmoset monkeys (Huff *et al.* 2001, Li *et al.* 2005, Mitchell *et al.* 2008).

After entering the gonadal ridge, germ cells home into their testicular stem cell niches at the basement membrane of seminiferous tubules early during development. These niches are microenvironments composed of cellular as well as molecular components, which regulate SSC self-renewal as well as the differentiation into haploid spermatozoa (van der Kooy *et al.* 2000, Spradling *et al.* 2001). Importantly though, differences in the SSC systems in rodents and men make the existence of different niche types likely. Characteristic of the SSC system in mice is that the A_{single} spermatogonia, which include the stem cell population, give rise to large cell clones via numerous mitotic divisions (Ehmcke *et al.* 2006).

In contrast to that, the adult SSC system in primates contains A_{dark} spermatogonia, which are considered the mitotically quiescent reserve, a cell population which does not exist in the rodent system. Additionally, the SSC system in primates contains the A_{pale} spermatogonia, which actively proliferate thereby preserving the stem cell pool or give rise to B spermatogonia (Ehmcke *et al.* 2006). In mice, functional role of individual growth factors have been demonstrated for regulation of SSC self-renewal and maintenance (Meng *et al.* 2000, Chen & Liu 2015). Even though, expression of individual growth factors or their respective receptors can be detected in mice as well as in men, different niches are required for support of respective SSC systems. In particular, Sertoli cells are an important component of the SSC niche, as they form the compartments within the seminiferous tubules and provide physical contact to germ cells at different stages of germ cell differentiation. Moreover, Sertoli cells provide essential growth factors including glial cell line-derived growth factor (Meng *et al.* 2000, Hofmann 2008), fibroblast growth factor 2 (Mullaney & Skinner 1992, Goriely *et al.* 2003) and Ets variant 5 (ETV5) (Sette *et al.* 2000, Hess *et al.* 2006, O'Bryan *et al.* 2012). A number of studies have identified GDNF as a crucial factor regulating self-renewal and maintenance of SSCs (Meng *et al.* 2000). Finally, the transcription factor ETV5, which helps to retain stem cells within their niches, was demonstrated to regulate expression of chemokine genes including *Cxcl12* in somatic Sertoli cells (Yoon *et al.* 2009, Chen *et al.* 2015). The role of this chemokine and its contribution to the SSC niche will be outlined in the following.

Expression pattern indicates a continuing role of the CXCL12 system throughout germ cell development

A functional role of the CXCL12 system for directed migration of early germ cells has been demonstrated in zebrafish and mice. Prerequisite for a continuing role of the CXCL12 system throughout germ cell development is the expression of the chemokine and its receptors in the testis or the ability to reactivate their expression also at later developmental stages.

To assess whether CXCL12 is expressed in testicular tissues postnatally, transcript profiles were determined throughout postnatal development of mice (on days 1, 7, 14, 21 and >37). Interestingly, *Cxcl12* levels remained stable till day 21 and were highest in adult animals (>37 days). Expression was confirmed at the protein level and CXCL12 was specifically localized in the cytoplasm of Sertoli cells (Payne *et al.* 2010, Chen *et al.* 2015), the somatic cell type with the most direct interaction with the germ cell population.

What is more, transcripts of the receptors *Cxcr4* and *Cxcr7* were also detected at RNA level throughout mouse testicular development. The expression profile

was different from the chemokine, however, as relative expression levels of *Cxcr4* and *Cxcr7* continuously increased from day 1 onwards and reached highest levels in animals aged >37 and 21 days, respectively. Requirement for a CXCL12-induced effect on the germ cell population is the expression of respective receptors on the cell surface of spermatogonia. For CXCR4, relevant studies have demonstrated that spermatogonia isolated via the cell surface receptor THY1, display higher expression levels of CXCR4 compared to testicular somatic cells (Oatley *et al.* 2006, Niu *et al.* 2016). More importantly even, CXCR4 protein was localized to the cell surface of spermatogonia residing along the basement membrane in testicular tissues from 3 and 5 days old as well as adult mice (Payne *et al.* 2010, Westernströer *et al.* 2014, Chen *et al.* 2015, Niu *et al.* 2016). Upon induction of meiosis (days 14 and 21 pp), expression of CXCR4 was also detected in differentiating germ cells as well as in spermatids and even interstitial cells in adult testes. Slightly controversial results were only obtained in one study, which reported the expression of the protein in spermatogonia and also in the somatic Sertoli and interstitial cells of the adult testis (Yoon *et al.* 2009).

Surprising data were obtained regarding the localization of CXCR7 in mice. In zebrafish, this receptor is localized to somatic cells during PGC migration and rather functions as a decoy receptor for Cxcl12 (Boldajipour *et al.* 2008). This alternative receptor CXCR7 displays a germ-cell-specific expression pattern in mice with a specific expression in gonocytes (day 1 pp) and spermatogonia at the basement membrane at subsequent developmental time points (days 7, 14 and 21 pp). Intriguingly though, co-stainings did neither lead to detection of cells co-expressing the spermatogonial marker LIN28a and CXCR4 nor CXCR7 and CXCR4 (Westernströer *et al.* 2014), indicating distinct populations of mouse spermatogonia with hitherto unknown properties.

Taken together, available literature demonstrates an evolutionarily and developmentally conserved expression profile of CXCL12 and its receptor CXCR4. This lends confidence to the assumption that the CXCL12 system remains functionally active even postnatally. However, the germ cell-specific expression pattern of CXCR7 suggests that the function of this receptor in mice may go beyond that of a decoy receptor.

To assess if this system is also conserved during testicular development in primates, localization studies have been performed employing the marmoset monkey as non-human primate model. This model has the advantage that it shares many similarities with the human, regarding its spermatogonial stem cell system as well as postnatal developmental phases (Albert *et al.* 2010). In marmosets, CXCL12 is constitutively expressed in the cytoplasm of Sertoli cells of infantile (8 and 16 wpp), prepubertal (32 wpp) and pubertal

(52 wpp) animals. Evidence for continued expression also in adult testis comes from marmoset, the macaque (*Macaca fascicularis*) as well as the human (Gilbert *et al.* 2010, McIver *et al.* 2013, Westernströer *et al.* 2015, Tröndle *et al.* 2017). Data on the expression pattern of the receptor CXCR4, however, is less complete. While data are hitherto not available for marmoset monkey, in human testicular tissues, CXCR4 was detected in all germ cell stages, with the exception of mature sperm (Gilbert *et al.* 2010, McIver *et al.* 2013). It is of note though that an independent study rather reported an expression of CXCR4 in interstitial leukocytes and macrophages of testicular tissues (Habasque *et al.* 2002). Reasons for this inconsistency remain hitherto elusive. Comprehensive and consistent data have been published though for the expression profile of the alternative receptor CXCR7. During postnatal testicular development of the marmoset (16, 32, 52 and >80 wpp) and human (2 weeks and 6 months) CXCR7 is expressed by a subset of gonocytes, pre-spermatogonia and spermatogonia. This expression pattern persisted as CXCR7 showed a specific expression in pre-meiotic cells located at the basement membrane in adult tissues (Westernströer *et al.* 2015).

Based on current data, the expression pattern of CXCL12 and its receptors appears to be highly conserved in mice and primates including men (Fig. 1). Current data demonstrate that expression of CXCL12 and its receptors is retained throughout testicular development, and it has been suggested that this system may also allow for directed germ cell migration in the adult testis. Although spermatogonia may not undergo extensive migration during tissue homeostasis, it is conceivable that gonadotoxic insults resulting in a depletion of spermatogonia necessitate migration of SSCs for the recolonization of seminiferous tubules and re-establishment of spermatogenesis. In this case, the CXCL12 system would act as a safeguard for male fertility. Prerequisite for this function are the persistent ability of adult SSCs to migrate towards CXCL12. To test this functional property, *in vitro* as well as *in vivo* migration assays have been performed using mice as animal models.

CXCL12-induced migration of adult mouse germ cells/SSCs *in vitro* and *in vivo*

First evidence that undifferentiated germ cells maintain their ability to respond to a CXCL12 gradient came from *in vitro* assays. The Boyden-chamber assay was employed as *in vitro* system and revealed that three times more mouse SSCs migrated in response to CXCL12 (10 ng/L) compared to controls. This effect was abolished by addition of AMD3100, a selective chemical antagonist of CXCR4. These *in vitro* data strongly suggested that directed migration also of adult murine SSCs is regulated in a CXCL12-dependent fashion (Niu *et al.* 2016).

Figure 1 Localization of CXCL12, CXCR4 and CXCR7 in seminiferous tubules of mice, marmoset monkeys and humans. CXCL12 (blue) is expressed in the cytoplasm of Sertoli cells of all species. CXCR4 (orange) expression was detected in cells located at the membrane of seminiferous tubules in mice, whereas in human tissues, the receptor is expressed in all germ cells except for mature sperm. No data are available for the CXCR4 expression in marmoset testicular tissues. Expression of CXCR7 (green) was determined in spermatogonia in adult testes of all three species. ES, elongated spermatids; SC, Sertoli cells; SPC, spermatocytes; Spd, spermatids; Spg, spermatogonia.

To unravel the potential role of the CXCL12 system for SSC migration and homing *in vivo*, a modified germ cell transplantation assay has been employed. The original germ cell transplantation assay was established in 1994 and entails the transplantation of donor cells into seminiferous tubules, devoid of germ cells (Brinster & Zimmermann 1994). Following transplantation, SSCs can migrate from the lumen of the seminiferous tubules towards the basement membrane, where they colonize empty niches and re-establish spermatogenesis (Nagano *et al.* 2000). This assay thereby provides functional proof for the presence of SSCs in a cell population. More recent studies have used modification of the SSC niches to unveil those factors that promote or prevent homing of SSCs into their niches. Whereas the artificially increased expression of rat GDNF prior to germ cell transplantation did not result in increased SSC colonization, increased CXCL12 expression led to a 2-fold increase in SSC colonies (Kanatsu-Shinohara *et al.* 2012). These findings were corroborated by independent studies demonstrating that the transplantation with AMD3100 along with the germ cell suspension resulted in significantly lower numbers of donor-derived SSC colonies. A comparable effect was observed following repeated administration of AMD3100 following germ cell transplantation (Niu *et al.* 2016). These analyses demonstrated the specific role of CXCL12/CXCR4 interaction for SSC homing also in adult mouse testes and indicate a role of the CXCL12 system for tissue regeneration (Fig. 2). To date, no functional data are available for primates or the human, but it appears likely that the same mechanisms also apply in these species. As the CXCL12 concentration had been artificially increased in the outlined experiments, a natural increase

of CXCL12 in the testis upon germ cell loss is required for this system to be of functional relevance.

Response of niche-associated factors to gonadotoxicity in mice and primate testes

Germ cell loss and associated emptying of SSC niches in mice can be induced by busulfan which exerts

Figure 2 The role of the CXCL12 system in the homing process of SSCs into their niches. The process of SSC homing involves the formation of a CXCL12 gradient by Sertoli cells. SSCs then migrate along this gradient and home into their niches.

its cytotoxic effect preferentially on spermatogonia, resulting in a successive loss also of the more differentiated germ cells (de Rooij & Kramer 1968). Following treatment with busulfan at 38 mg/kg, loss of LIN28a-positive spermatogonia was obvious on day 7 after treatment, whereas more differentiated germ cells (DDX4-positive) persisted until day 21. Importantly, on day 28 after treatment, repopulation of individual seminiferous tubules by LIN28-positive spermatogonia was observed (Westernströer *et al.* 2014). Although this sequence of events is corroborated by other studies, it is of note that the time of spermatogenic recovery is dependent on the treatment dose (Zohni *et al.* 2012). Nonetheless, studies using mice as animal model assessed the response of the testicular somatic cells in the first days following cytotoxic treatment and found comparable effects. In particular, Sertoli cells showed a rapid response with an upregulation of *Gdnf* following 15 mg/kg and 30 mg/kg of busulfan treatment (Zohni *et al.* 2012). In line with this, an independent study analysed 6 niche-associated factors and found comparable results with a significant upregulation of all 6 factors including *Gdnf* and *Cxcl12* (Westernströer *et al.* 2014). As testicular tissue composition is still comparable to control tissues, these findings indeed suggest a prompt and temporary response of testicular somatic cells upon SSC depletion and tissue damage. Although protein data are largely lacking, a number of possible reasons for this findings have been proposed. In particular, increased levels of GDNF may enhance the self-renewal activity of remaining SSCs thereby enabling the repopulation of seminiferous tubules. Upregulation of CXCL12 may function as an enhanced homing signal guiding newly formed SSCs into vacant niches (Oatley *et al.* 2007, Wu *et al.* 2011, Yang *et al.* 2013). This assumption is supported by the observation that out of 6 analysed niche-associated factors only *Etv5* and its downstream target *Cxcl12*, showed significantly higher expression levels also on day 28 following treatment. At this time point, early spermatogenic repopulation was observed a process that might be supported by increased levels of CXCL12 (Westernströer *et al.* 2014). Using macaque as non-human primate model, effect of gonadotoxic insult on testicular somatic cells has also been evaluated. In particular, protein expression patterns of CXCL12 were analyzed in testicular tissues, following irradiation with 1 or 4 Gy, respectively. Comparable to busulfan treatment, irradiation with these doses induces germ cell depletion but still allows for the recovery of spermatogenesis (1 Gy: 75–200 days; 4 Gy: 3–4 years) (van Alphen *et al.* 1988, Jahnukainen *et al.* 2007). Using a xenografting model, the effect of this gonadotoxic insult on the stem cell niche of prepubertal macaques was assessed. Testicular tissues were retrieved 6.5 months following graft transplantation, and histological evaluation unveiled that spermatogonial re-population had progressed further in tissues treated with 1 Gy

compared to those treated with 4 Gy. These tissues were further evaluated for the expression pattern of CXCL12 at protein level. Quantitative analyses revealed that a significantly higher number of seminiferous tubules showed expression of CXCL12 in all Sertoli cells in the 1 Gy compared to the 4 Gy group (Tröndle *et al.* 2017). These results demonstrate that the upregulation of CXCL12 in response to spermatogonial loss induced by gonadotoxic insult is evolutionarily conserved in mice and primates (Zohni *et al.* 2012, Westernströer *et al.* 2014) and suggests an involvement of the chemokine CXCL12 in spermatogonial repopulation. It also appears likely that CXCL12 may not only play a role in the stimulation of germ cell migration and homing but also stimulates spermatogonial proliferation.

CXCL12 does not enhance germ cell proliferation in zebrafish, mice or human

The process of germ cell migration is characterized by an increase of the PGC population by mitotic expansion. CXCL12 has therefore been proposed to act as an external signal supporting the proliferation of PGCs. However, data from zebrafish lacking *Cxcl12* demonstrate that this chemokine is not required for PGC proliferation (Wei & Liu 2014). In mice, insights were gained by evaluation of CXCR4-deficient embryos, which displayed a strikingly lower number of PGCs by day E11.5. However, comparative quantification of PGCs during the developmental stages from 10.5 to 12.5 revealed that CXCL12/CXCR4 interaction was dispensable for PGC proliferation but required for germ cell survival (Ara *et al.* 2003).

It has been speculated that the interaction of CXCL12 with its receptor CXCR4 might also be crucial in the adult testis for SSC proliferation. In order to address a potential influence of CXCL12 on SSC proliferation mouse SSC cultures were treated in parallel approaches with recombinant CXCL12 at a concentration of 10 ng/mL, with AMD3100 or a combination of both. Unexpectedly though, the numbers of SSCs were comparable in all treatment groups following 7 days of culture (Niu *et al.* 2016). Comparable results were obtained in an independent study. Following 6 days of culture, the numbers of germline stem cells were similar irrespective of whether CXCL12 or AMD3100 had been added to the culture medium (Kanatsu-Shinohara *et al.* 2012). These findings consistently show that there is no effect of the CXCL12/CXCR4 interaction on SSC proliferation. Although the influence of CXCL12 on SSC proliferation in primates has not been assessed directly, data are available for the human testicular germ cell tumour-derived cell line TCam-2 (Gilbert *et al.* 2010, McIver *et al.* 2013). As mentioned previously, and in accordance with data obtained for mouse spermatogonia, CXCL12 had no effect on the proliferation of TCam-2 cells *in vitro* (Gilbert *et al.* 2010, McIver *et al.* 2013).

Figure 3 Functional analyses of CXCL12, CXCR4 and CXCR7 in rodents and primates including the human. (A) In mice, current data demonstrate that CXCL12 has an effect on germ cell migration *in vitro* and *in vivo* and in particular for homing of SSCs into their niches. Furthermore, analyses of murine testicular tissues following gonadotoxic insult (radiation or chemotherapy) and in particular in tissues showing repopulation of spermatogonia revealed increased expression levels of CXCL12. However, CXCL12 does not have an impact on germ cell proliferation. (B) For primate testicular tissues, analyses showed comparable results to rodent studies regarding germ cell migration and proliferation and an activation of the CXCL12 system following gonadotoxic insult. However, in primates, *in vivo* germ cell transplantation studies have not yet been performed. Positive effect of CXCL12 in the different processes is indicated by an arrow, whereas a line demonstrates the absence of an effect.

Conclusion and future perspectives

Available data clearly demonstrate that the function of CXCL12 interaction with its receptors CXCR4 and CXCR7 is essential for directed migration of PGCs in zebrafish and mice. Based on this evolutionary conservation, it is likely that the same is true also for primates, including the human. As access to human testicular tissues from early developmental stages is limited for ethical and legal reasons, expression pattern of CXCL12 and its receptors needs to be assessed during early developmental stages in primate animal model, to gain insight if this system is also regulating PGC migration. Moreover, more recent studies have demonstrated that the CXCL12 system remains functional also at later stages of germ cell development. In particular the homing process of mouse gonocytes into their germ cell niches is regulated by the CXCL12 system. But what is more, expression of CXCR4 and CXCR7 on the cell surface persists even in adult spermatogonia and facilitates their CXCL12-induced migration. For adult mouse SSCs, the process of SSC-homing following germ cell transplantation has been demonstrated to be CXCL12 dependent. Evolutionarily highly conserved expression pattern of CXCL12 in Sertoli cells of mice, marmoset and human indicate that this chemokine is also an essential part of the adult SSC niche. For primates and humans, functional *in vitro* or *in vivo* data are not yet available (Fig. 3). As a first step, ability of primate or human SSCs to migrate towards a CXCL12 gradient can be tested using the Boyden chamber approach, provided that sufficient cell numbers can be obtained for subsequent analyses. For final functional proof, germ cell transplantation assays

need to be performed. As germ cell transplantation assays were shown to be feasible also employing adult primate SSCs as well as the human TCam-2 cell line (Nettersheim *et al.* 2012, Langenstroth *et al.* 2014), transplantation into testicular tissues with artificially increased expression levels of CXCL12 would provide evidence on the regulatory mechanisms of SSC migration and homing in the primate species.

Declaration of interest

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