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The migration and loss of human primordial germ stem cells from the hind gut epithelium towards the gonadal ridge

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ABSTRACT Human primordial germ cells (PGCs) can be recognized in the yolk sac wall, from 3-4 weeks post conception (wpc), in the hind gut epithelium from week 4 and in the gonadal area from early week 5. The objective of this study was to map the migration route of PGCs and elucidate the role of the nervous system in this process. Sixteen human specimens, 5-14 wpc obtained from legal abortions were included. On serial paraffin sections, PGCs were detected immunohistochemically by expression of OCT4 and c-Kit, nerve fibers by β-III-tubulin and stem cell factor (SCF) as a possible chemoattractive cue for PGC migration. PGCs were present in the hind gut epithelium, in the mesenchyme of the dorsal mesentery and in the developing gonadal ridge of 4-6 wpc embryos, prior to connections between the enteric and the sympathetic nervous system. From 6 wpc onwards, the PGCs travelled along the developing nerve fibers from the wall of the hind gut via the dorsal mesentery to the midline of the dorsal wall and laterally into the gonads. Numerous PGCs were still present in the nervous system by 14 wpc. PGCs in 4-5 wpc embryos are suggested to leave the gut epithelium by EMT-like transition. SCF may facilitate further migration, but after establishment of connections between the enteric and sympathetic nervous systems. PGCs follow sympathetic nerve fibers towards the gonads. PGCs failing to exit the nerve branches at the gonadal site, may continue along the sympathetic trunk ending up in other organs where they may form germ cell tumors if not eliminated by apoptosis.

KEY WORDS: *germ cell, migration, nerve fiber, human embryo, first-trimester pregnancy, germ cell tumor*

Introduction

Human primordial germ cells (PGCs) are the precursors of the germ cell line and are believed to originate in the endoderm of the yolk sac wall near the allantois evagination where they are recognized from three to four weeks post conception (wpc) (Witschi, 1948). In the fundamental work of Witschi the origin of PGCs are suggested to be either endodermal or arise from primitive stem cells, which also are the source of the endoderm (Witschi, 1948). Later studies in mouse suggests that the PGC lineage derives from the very proximal epiblast (i.e. embryonic ectoderm) and not from the endoderm (McLaren, 2003; Stallock *et al.,* 2003). Apart from giving rise to the germ line, epiblast cells also give rise to extraembryonic structures such as allantois, yolk sac mesoderm, and to both layers of the amnion (Lawson *et al*., 1999). The specification of the pluripotent epiblast cells to PGCs is determined by signals from the extraembryonic tissues (Ohinata *et al*., 2005). Specification of the PGC precursors in mouse occur in the yolk sac near allantois and is initiated by the expression of *Blimp-1,* followed by expression of PGC markers such as *Stella*, c-Kit, OCT4 and SCF, which are all involved in the survival and migration of the PGCs (Gu *et al.,* 2009; Hayashi *et al.,* 2007; Chang *et al*., 2002). However, the network of signals that controls the specification and migration in human is poorly understood. PGCs have been described to migrate from the dorsal mesentery of the hind gut during the fourth to fifth wpc. From there they invade the developing gonadal ridge along the ventral part of the mesonephros around five wpc (Witschi, 1948). Using advanced technologies cultured mouse PGCs have been filmed while migrating from the hind gut, along the body wall to the gonadal ridge, suggesting that PGCs are actively motile

Abbreviations used in this paper: CNS, central nervous system; CRL, crown-rump length; EMT, epithelial-mesenchymal transition; ENS, enteric nervous system; GCT, germ cell tumor; NCC, neural crest cell; pc, post conception; PGC, primordial germ cell; SCF, stem cell factor; SNS, sympathetic nervous system; wpc, weeks post conception.

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(Molyneaux *et al.,* 2001). PGCs have been observed in different tissues along the migration route to the gonad. Some PGCs have been found within the mesentery while others have been identified in the genital anlage or near the dorsal aorta, which may indicate that migrating PGCs have varying migration speeds and patterns (Makabe and Motta, 1989). During migration in the hind gut mesentery the PGCs appear for a short time to extend long processes linking these cells to each other (Molyneaux *et al.,* 2001). To what extent the PGCs migrate as individual single cells or as a connected group remain largely unknown as does the molecular basis controlling the migration.

Several mechanisms concerning PGCs migration to the gonad have been suggested. Fibronectin is present along the migratory pathway at the time of migration and has been suggested to play an important role in the translocation of PGCs (Fujimoto *et al.*, 1985). SCF binding to the tyrosine kinase receptor c-Kit located on the PGCs surface may facilitate migration and colonization of the gonadal ridge (Runyan *et al.,* 2008; Hoyer *et al.,* 2005; Godin *et al.*, 1991). The PGCs response to SCF may be potentiated by the protein Ror2 expressed by the PGCs. Ror2 is suggested to increase polarity of the cell, which is needed for cells undergoing directed migration (Laird *et al.,* 2011). Chemotactic substances may also guide PGCs to the gonad (Byskov and Høyer, 1994; Witschi, 1948) and adhesion molecules present on the PGCs have been described to play an important role in their migratory ability (De Felici *et al.,* 2005; Bendel-Stenzel *et al.,* 2000; Anderson *et al.,* 1999). Whether PGCs are translocated from the yolk sac to the gonads mainly by active migration has been questioned, and it has been suggested that the translocation is a combination of active movement and tissue modulation during embryogenesis (Freeman, 2003; Wrobel and Suss, 1998; Witschi, 1948). In the human embryo the dorsal part of the yolk sac containing the PGCs, becomes incorporated in the developing gut during the lateral folding in week 4 pc, indicating that the PGC translocation into the gut may be passive (Freeman, 2003). Either way, questions are left open concerning how the PGCs are displaced at this early stage.

In the human embryo around 35 days pc it was recently shown that PGCs are present along autonomic nerve fibers and Swann cells from the dorsal mesentery to the gonad, where they seem to be delivered via fine nerves (Mollgard *et al.,* 2010; Hoyer *et al.*, 2005). The migration is accompanied by a wave of SCF, expressed by the surrounding somatic cells, which is suggested to facilitate the migration and survival of the PGCs (Gu *et al.,* 2009). In the presence of SCF the PGCs close to the genital ridge continue to migrate and colonize the genital ridge, while PGCs remaining further away in the midline body structures normally die by the *Bax*-dependent apoptotic pathway in the absence of SCF (Gu *et al.*, 2009; Runyan *et al.,* 2006; Godin *et al.*, 1991). In later embryonic development SCF also functions as a survival factor and tends to eliminate PGCs in the body midline by its down-regulation (Runyan *et al.,* 2006).

Several studies of mammals have revealed the presence of PGCs in extragonadal tissues long after gonadal differentiation, showing that extragonadal PGCs may remain for some time. In fact PGCs that have been observed in the adrenal glands (Wrobel and Suss, 1999; Rich, 1995), not only survive but also, in both genders, differentiate and mature into oocytes (Upadhyay and Zamboni, 1982). Thus, in males, PGCs found in the adrenal glands follow the female pattern and differentiate into oocytes indicating that proper differentiation along the male line may require location in the testis (Upadhyay and Zamboni, 1982).

PGCs are suggested to have a pluripotent stem cell potential

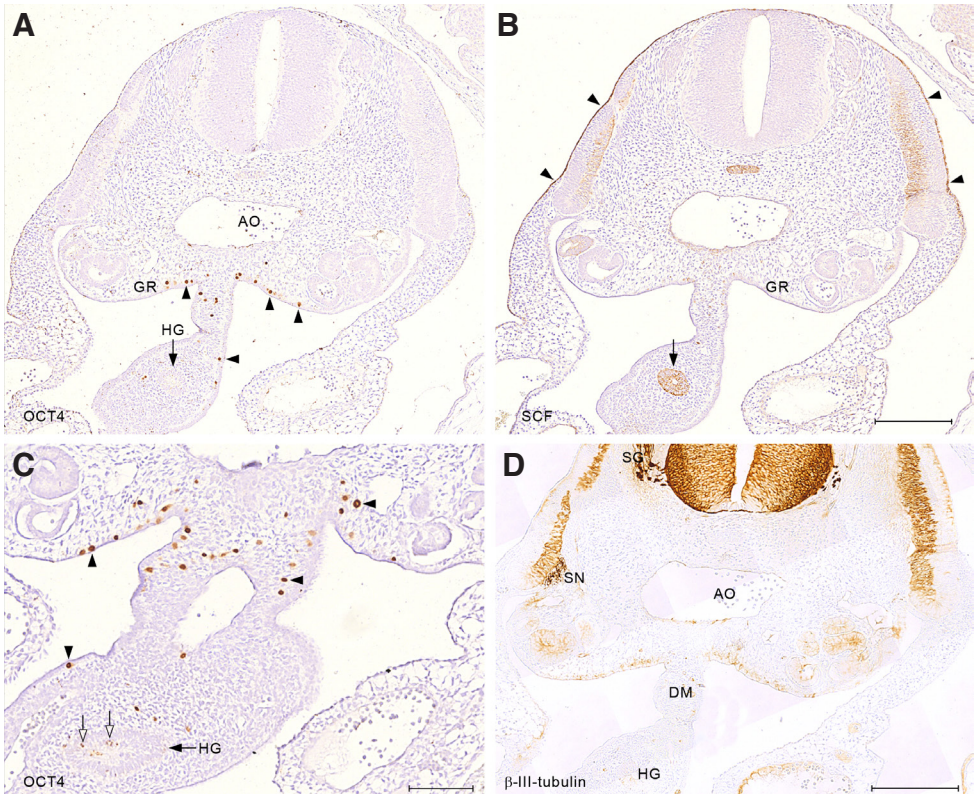

Fig. 1. Transverse section through the hindgut and dorsal mesentery of a human embryo, 4.5 wpc. *Transverse section through the hindgut and dorsal mesentery of a 4.5 week old human embryo, (CRL = 5 mm, 4 weeks and 4 days pc) showing immunohistochemical staining for OCT4* **(A,C)**, *SCF* **(B)** *and β-III-tubulin* **(D)**. **(A)** *Primordial germ cells show a distinct nuclear reactivity (arrowheads) on their way from the hindgut (HG) to the gonadal region (GR), where they initiate the formation of the gonads. PGCs are either associated with the mesothelium or migrating in the mesenchyme of the hindgut wall and the dorsal mesentery.* **(B)** *The hindgut epithelium (arrow) and the ectoderm (arrowheads) show a marked expression of SCF.* **(C)** *A section close to* **(A)** *in a bit higher magnification. Remnants of OCT4 reactivity (open arrows) are present in the hindgut epithelium (HG) and many PGCs are shown (arrowheads).* **(D)** *β-III-tubulin stains the developing spinal ganglion (SG) and the spinal nerve (SN). No staining of the para- and preaortic sympathetic ganglia, which later will be situated lateral and ventral to aorta (AO). No nerve fibers are found in the hindgut (HG) or the dorsal mesentery (DM). A weak staining of the gonadal and mesonephric area is present. S*cale bars: *200 μm (A,B,D); 100 μm (C).*

that may give rise to hematopoietic stem cells, which originates intraembryonic in the aorta-gonad-mesonephric region (Dzierzak *et al.,* 1997). PGCs have also been found in rich numbers in the para-aortic tissue, liver and branchial arches in the pig (Wrobel and Suss, 1998). In humans, primary sites of ectopic germ cells are within the CNS but they are also seen in the pelvic region, the mediastinum and thorax (Arora *et al.,* 2012).

Teilum has proposed a theory concerning the malignant potential of extragonadal germ cells known as the 'germ cell theory', suggesting that extragonadal germ cell tumors (GCTs) originate from stray PGCs, which have undergone a malignant transformation during embryonic development (Teilum, 1976; Teilum, 1965). One established way of systemizing GCTs is based on the observations by Teilum, who favoured the idea that germ cells may give rise to germinomas, which includes GCTs in the gonads and tumors composed of totipotent cells that produce embryonic carcinomas. These in turn give rise to yolk sac tumors, choriocarcinomas and teratomas. This idea has remained the basis in the WHO classification of GCTs today (Louis *et al*., 2007). In the human malignant GCTs may arise at all ages. In pre-pubertal humans the incidences only account for 3% of all malignant tumors increasing to 14% in pubertal young adults. The majority of all postnatal GCTs are found in testis, which account for 93% of all GCTs. Ovarian and extragonadal GCTs represent only 4% and 3% of all GCTs, respectively, with the CNS being the most common extragonadal site for GCTs accounting for almost half of all extragonadal tumors (Arora *et al.,* 2012; Smith *et al.,* 2006). The large difference in the incidence of testicular and ovarian/extragonadal GCTs reported might be related to the different developmental pattern between male and female germ cells. In the ovary all PGCs become oocytes by undergoing meiosis early in life whereas in the testis spermatogonia continue to proliferate throughout life (Byskov, 1982; Baker, 1963), giving rise to a much greater GCT potential in the testis. The PGCs preference to the rostral-caudal part of the sympathetic nervous system (SNS) may explain the specific midline distribution of extragonadal germ cell tumors.

The aim of this study is to describe the migration routes that the PGCs follow from the hind gut epithelium to the gonad and the role of the nervous system in this process.

Results

Detection of PGCs and nerves in embryos of 4-5 wpc

OCT4 shows a distinct nuclear reactivity in PGCs in 4-5 wpc embryos. PGCs are observed in the hind gut, the gut mesentery, and in the coming gonadal region (Fig. 1 A,C). The migrating PGCs are often associated with the mesothelial lining, the mesenchyme of the hind gut wall and the dorsal mesentery. Remnants of some of the PGCs within the hind gut epithelium are faintly stained as seen in a week older embryo as well (Fig. 2E). The hind gut epithelium and the ectoderm show marked

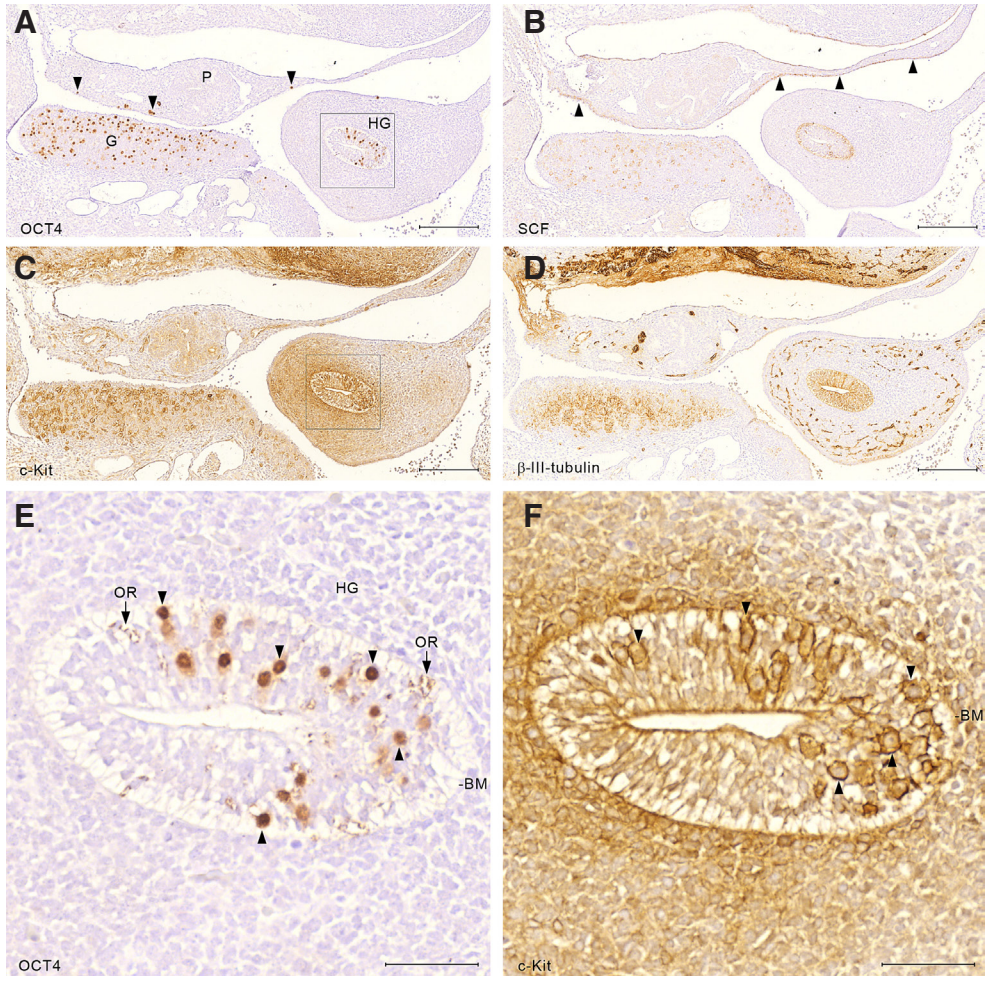

Fig. 2. Sections through the abdomen of a human embryo, 5.5 wpc. *Sagittal section through the abdomen of a 5.5 wpc human embryo, (CRL = 9 mm, 5 weeks and 4 days pc) showing immunohistochemical staining for OCT4* **(A, E)**, *SCF* **(B)**, *c-Kit* **(C,F)** *and β-III-tubulin* **(D)**. **(A)** *Transverse section of the hindgut (HG), gonad (G), and pancreas (P). Many PGCs are present within the gonad and the HG epithelium. PGCs are also present in the dorsal part of the mesentery within the mesothelial lining of the pancreas (arrowheads).* **(B)** *Arrowheads indicate SCF stained PGCs in the mesothelium. PGCs of the hindgut are stained. Many faintly stained PGCs are also present in the gonad.* **(C)** *c-Kit is also expressed in the gonad and the hindgut (framed area), shown in higher magnification in* **(F)**. **(D)** *Nerve fibers of the ENS are now present also in the hindgut as shown by β-III-tubulin reactivity. Some staining is also present in the gut epithelium and in the center of the gonad.* **(E)** *Higher magnification of the framed area in* **(A)** *PGCs nuclei are indicated by arrowheads. Fragments of OCT4 reactivity (OR) and lack of a distinct basement membrane (-BM) are marked.* **(F)** *Cell membrane and cytoplasm of PGCs (arrowheads) are strongly stained for c-Kit. In small segments of the epithelium the basement membrane seems to be absent (-BM). S*cale bars: *200 μm (A,D); 50 μm (E,F).*

expression of SCF (Fig. 1B). At this developmental stage the spinal nerve and the developing spinal ganglion are strongly stained for β-III-tubulin characteristic for nerve fibers (Fig. 1D). The para- and preaortic sympathetic ganglia lateral and ventral to aorta are not yet visible by β-III-tubulin staining. Neither are nerve fibers in the hind gut and the dorsal mesentery. However, a slight but distinct β-III-tubulin staining in the gonadal ridge and the adjacent mesonephric tissues are recognized (Fig. 1D).

Detection of PGCs and nerves in embryos of 5-6 wpc

In the 5½ wpc old human embryos many OCT4 stained PGCs are present in the hind gut, the mesentery and in the gonads (Fig. 2 A,E). Some PGCs of the gut epithelium exhibit a solid nuclear staining, whereas others show a rather fragmented pattern when located close to the basement membrane of the epithelium (Fig. 2E). However, at these places the basement membrane appears to be absent (Fig. 2 F,E). PGCs are also observed in the mesothelium lining the pancreas (Fig. 2A). c-Kit is expressed in the gonads, the hind gut and pancreas and in many other organs (Fig. 2C). In the hind gut nerve fibers of the enteric nervous system are present as shown by β-III-tubulin reactivity (Fig. 2D). However, also reactivity in the ovary and in the epithelium of the gut is observed.

Detection of PGCs and nerves in embryos and fetuses of 7-14 wpc

At these ages the splanchnic nerve fibers extend from the dorsal abdominal body wall via the dorsal mesentery towards the enteric nervous system in the intraperitoneal parts of the gastrointestinal system, as visualized by β-III-tubulin reaction (Fig. 3 A,B). Thus, a 'nervous freeway' between stomach, duodenum, pancreas, ileum and colon, and the pre- and paraaortic parts of the sympathetic nervous system is created. Along or in between the nerve fibers, small, rounded, unstained holes are seen (Fig. 3B). These holes probably correspond to PGCs (Fig. 3C). A similar picture is seen in 14 wpc fetuses where some c-Kit stained PGCs are still associated with nerve fibers (Fig. 3D), although the number of PGCs may be lower in the older fetus than in the younger embryo at 7 wpc (Fig. 3C).

Confocal microscopy

The simultaneous presence of c-Kit-positive PGCs associated with β-III-tubulin-positive nerve fibers is clearly demonstrated by means of confocal microscopy of a human embryo aged 7 weeks and 6 days pc (Fig. 4). At this stage, connectivity is established between the enteric- and the sympathetic nervous system, visualized by positive β-III-tubulin reactivity (Fig. 4 B-C). Sympathetic nerve fibers are β-III-tubulin positive in the adrenal glands, the pancreas, and in particular in the dorsal mesentery. Positive β-III-tubulin reactivity is also seen in nerve fibers of the enteric nervous system (ENS), and especially in the plexus myentericus. Further, c-Kit reactivity is observed in the interstitial cells of Cajal of the plexus myentericus(see arrows in Fig. 4C) and at higher magnification a dense reactivity is seen in smaller mast cells without any contact to the nerve fibers (Fig. 4 D,F), previously identified and described using the same material (Høyer *et al.*, 2005). The large PGCs located in the periphery of the β-III-tubulin positive nerve fibers of the SNS demonstrate strong membrane-associated c-Kit reactivity (Fig. 4 D,F).

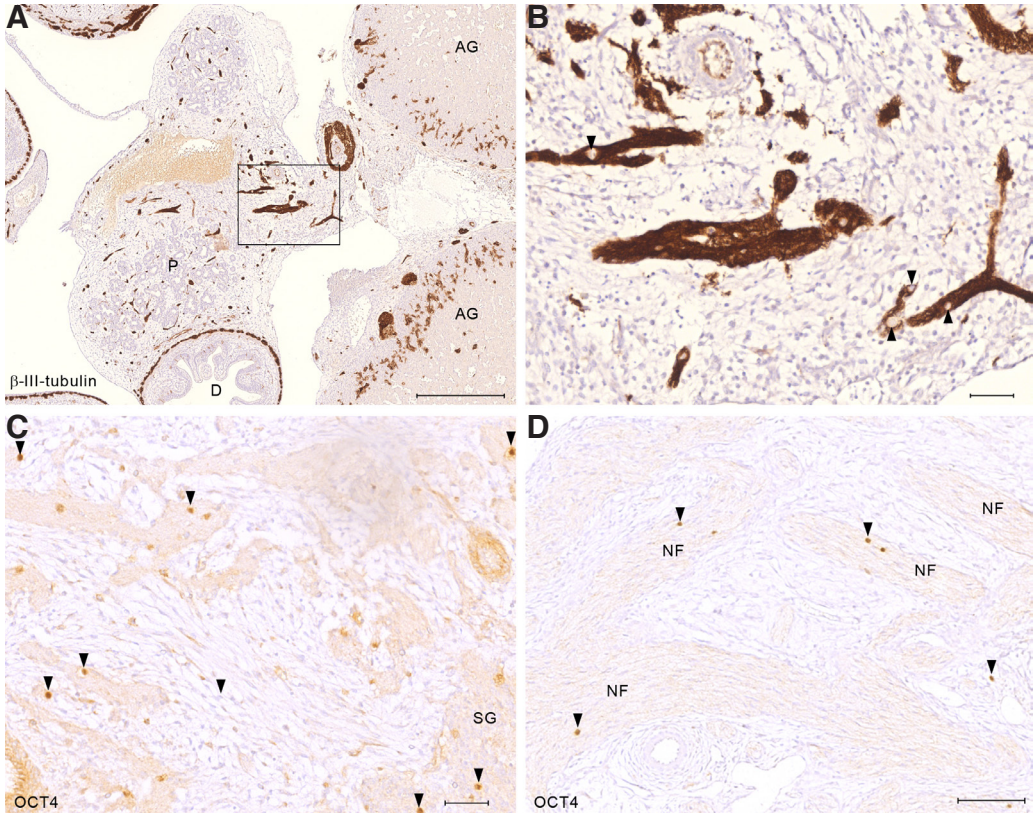

Fig. 3. Horizontal section through abdomen of a human embryo, 8 wpc and a human fetus, 14 wpc. *Horizontal section of human embryo, CRL = 30 mm, 7 weeks and 6 days pc in* **(A-C)**, *and of a human fetus, CRL= 103 mm, 14 weeks pc in* **(D)**. **(A,B)** *stained for β-III-tubulin and* **(C,D)** *for OCT4. Another section from the embryo in* **(A-C)** *will be described in details in Fig. 4.* **(A)** *β-III-tubulin positive splanchnic nerve fibers extend from the dorsal abdominal body wall via the dorsal mesentery towards the enteric nervous system in the intraperitoneal parts of the gastrointestinal system thereby creating a 'nervous freeway' between stomach, duodenum, pancreas, ileum and colon and the pre- and paraaortic parts of the sympathetic nervous system.* **(B)** *Higher magnification of the framed area in* **(A)**. *The large holes (arrowheads) correspond to PGCs.* **(C)** *Section close to* **(B)** *with OCT4 stained PGCs (arrowheads) closely connected to the nerve fibers.* **(D)** *A 14 wpc fetus in which some PGCs (arrowheads) are still found associated with nerve fibers (NF) of the sympathetic nervous system. Scale bars: (A) 500 μm, (B,C) 50 μm, (D) 100 μm.*

Discussion

The present study provides new information concerning the early location of OCT4-positive human PGCs in the hind gut epithelium and their subsequent migration towards the early gonadal anlage in human embryos and fetuses from 4th to 14th wpc. Moreover, a possible explanation for development of germ cell tumors in the body midline during development is proposed.

During embryonic folding the PGCs are incorporated into the endodermal epithelium of the developing hind gut from the base of the allantois in the region of the forming hind gut (Anderson *et al.*, 2000; Lawson *et al.,* 1999; Bendel-Stenzel *et al.,* 1998; Witschi, 1948). From the hind gut they seem to achieve a more motile character and migrate from the gut epithelium to the mesenchyme of the gut wall perhaps via EMT, a process which converts compact and ordered epithelial cells into migratory mesenchymal cells (Kerosuo and Bronner-Fraser, 2012). However, the PGCs of the hind gut may not be typical epithelial cells and may be able to leave the epithelium using other mechanisms than EMT. The interesting observation of PGCs penetrating the basement membrane and leaving the gut epithelium was already described in the seminal work of Witschi in 1948 (Witschi, 1948). We find PGCs migrating in the gut mesenchyme already from around day 32 pc. At this early embryonic stage autonomic nerve fibers have not yet been established, which in later embryonic development are suggested to function as the primary migration route (Mollgard *et al.*, 2010). This indicates that the PGCs may start their migration from the dorsal mesentery towards the gonadal ridge without nerve guidance. At this developmental stage the migration may be mediated by chemotaxic mediators such as SCF (Gu *et al.*, 2009; Runyan *et al.,* 2006), a factor expressed along the migratory pathway. Witschi already made these observations in 1948 (Witschi, 1948), and observed the EMT-like process by which PGCs penetrate the basement membrane of the hind gut more than fifty years before the EMT was defined. Witschi also suggested that the mesenchymal migration of the PGCs was guided by chemoattraction, a theory that has been supported by several studies since (Laird *et al.,* 2011; Byskov and Høyer, 1994; Fujimoto *et al.*, 1985). However, Witschi did not observe the PGC migration along the nerve pathway: *"They are not travelling along a given path, nor do they follow recognizable leader structures such as blood vessels or nerves",* he suggested in his pioneering work of 1948. As the oldest embryo in the work of Witschi was only 8 mm CRL corresponding to 5 wpc and the modern methods of immunohistochemistry were not developed at the time, he did not observe the developing, thin nerve fibers along which the PGCs were associated.

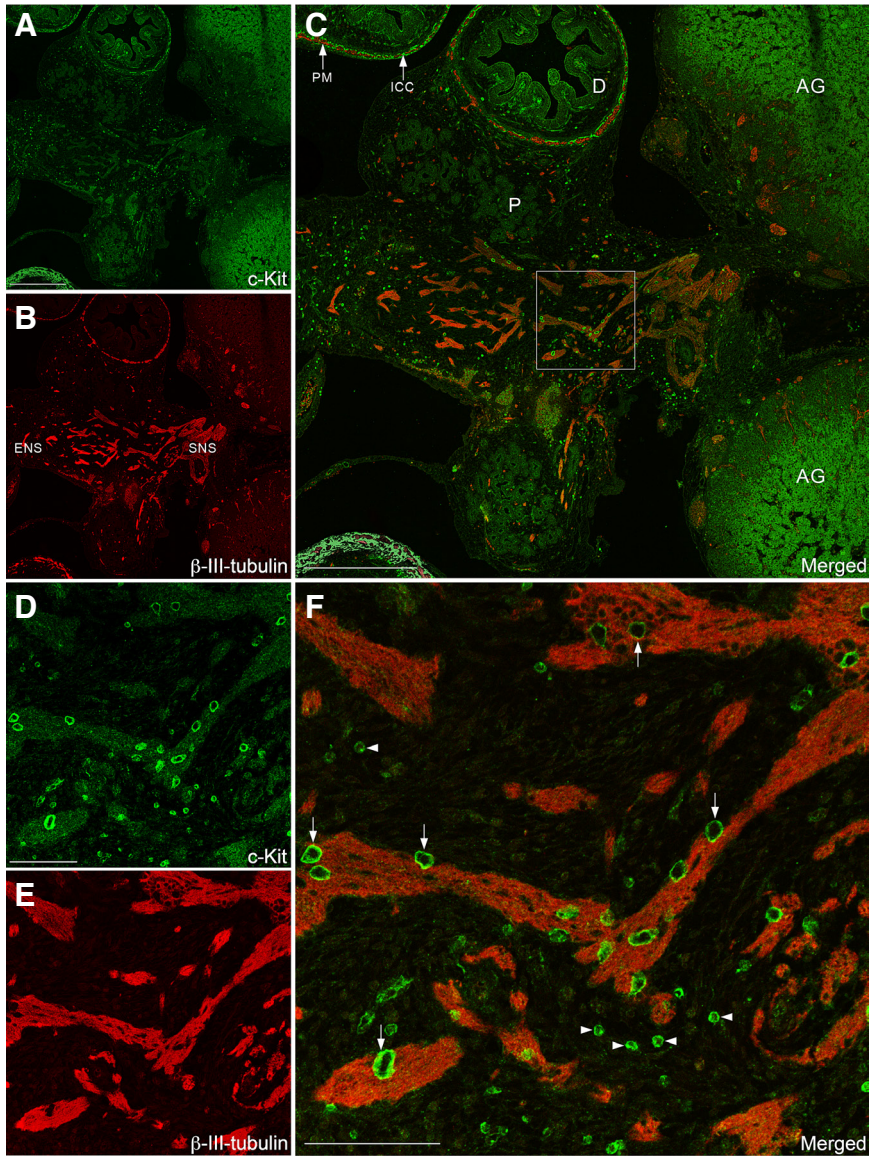

Fig. 4. Horizontal section through abdomen of a human fetus, 8 wpc. *Horizontal section of human embryo, CRL= 30 mm, 7 weeks and 6 days pc immunofluorescent labeled against c-Kit and β-III-tubulin antibody. Survey depicted* **(A-C)**, *with commencing connectivity of the enteric (ENS) and sympathetic (SNS) nervous system* **(B)**. **(C)** *Some sympathetic nerve fibers are found in the adrenal glands (AG), the pancreas (P), and especially in the dorsal mesentery located in the middle of the section. Positive β-III-tubulin reactivity is seen in nerve fibers of ENS, in general in the plexus myentericus (PM) and similar reactivity is observed in the duodenum (D). Furthermore, c-Kit is also observed in the interstitial cells of Cajal (ICC) of PM.* **(D)** *The PGCs in the nerve fibers demonstrate c-Kit reactivity.* **(E)** *Higher magnification of* **(B)** *demonstrating β-III-tubulin reactivity of SNS.* **(F)** *Higher magnification of boxed area in* **(C)**. *The larger PGCs, with strong membranous c-Kit reactivity are located in close correspondence to the periphery of the individual nerve fibers of the SNS (F, arrows). The small, densely labeled c-Kit-positive cells outside of the nerve fibers are mast cells (F, arrowheads). Scale bars: (A,B) 500 μm, (C) 200 μm, (D,E) 100 μm, (F) 50 μm.*

In 8 wpc embryos, the migration pattern has changed and c-Kit positive PGCs can now be seen along the developing autonomic nerve fibers as previously described (Mollgard *et al.*, 2010; Hoyer *et al.*, 2005). It is interesting to note that also mast cells stain for c-Kit and may be confused with PGCs. However, the PGCs are much larger than the mast cells, which can be distinguished from

PGCs by their expression of mast cell tryptase (Hoyer *et al.*, 2005).

The cells of the autonomic nervous system arise from neural crest cells (NCC) (Young *et al.*, 2011), a migratory cell population, which give rise to many diverse structures in the embryo such as the dorsal root-, vagal-, sacral-, and sympathetic ganglia (Kasemeier-Kulesa *et al.,* 2005; Wallace and Burns, 2005; Fu *et al.*, 2003). In both chicken and mouse the NCC, which form the sympathetic ganglia develop in the caudal hindbrain and migrate in a ventrolateral direction between the somites toward the dorsal aorta, where a reorganization takes place forming the sympathetic ganglia (Young *et al.*, 2011; Kasemeier-Kulesa *et al.,* 2005). In the pig, neurons from the sympathetic ganglia have been described to migrate laterally toward the gonadal ridge at day 21 pc, the same time as the first PGCs enter the gonadal anlage (Dees *et al.*, 2006). Furthermore in humans, NCCs have been reported to enter the foregut in week 4 pc and migrate rostral-caudally to reach the hind gut in 8th wpc (Wallace and Burns, 2005; Fu *et al.*, 2004). These findings support the results of the present study, which detects autonomous nerve fibers in the same time frame. Furthermore, we observe PGCs migrating along the developing sympathetic nerve fibers of the autonomous system.

Between days 29 and 32 pc, which is before connections between the ENS and the SNS can be detected, the migration may be guided by mediators such as SCF, which have been suggested to play an essential role in the migration and survival of PGGs (Runyan *et al.*, 2008; Hoyer *et al.*, 2005; Godin *et al.,* 1991). We also find SCF richly expressed along the migratory route. The early guidance is probably a symphony of many mediators, among others fibronectin (Fujimoto *et al.*, 1985) and adhesion molecules present on the PGCs (De Felici *et al.,* 2005; Bendel-Stenzel *et al.,* 2000; Anderson *et al.*, 1999).

PGCs are found not only along the sympathetic trunk and in the area of the gonads, but also long past the gonads in the abdomen, adrenal glands, heart, lungs and CNS. The PGCs are, however, normally eliminated by apoptosis (Gu *et al.*, 2009; Runyan *et al.*, 2008; Molyneaux *et al.*, 2001). Extragonadal germ cells, which fail to be eliminated, have a potential to form tumors. Interestingly, the most common locations of extragonadal GCTs are the abdomen, mediastinum, pineal gland and other median parts of the CNS (Arora *et al.*, 2012), which are innervated by sympathetic trunk nerve fibers (Nilsson, 2011). Thus, PGCs may end up in just these parts of the body because they fail to exit the sympathetic trunk at the gonadal site, and instead continue migration along other nerve branches from the sympathetic trunk.

The phenomenon of migrating PGCs disorientating along the way from the hind gut to the gonad has been given some attention in respect to the formation of extragonadal GCTs (Echevarria *et al.,* 2008; Oosterhuis *et al.*, 2007; Gonzalez-Crussi, 1982). It is interesting to note that GCTs most often are located along the midline of the body, an observation that has been explained by the migration route of PGCs during early embryonic development (Echevarria *et al.*, 2008; Gonzalez-Crussi, 1982). The observations by Møllgård *et al.,* (Mollgard *et al.*, 2010) refine this picture by suggesting that PGCs migrate from the dorsal mesentery to the gonadal ridge along autonomic nerve fibers. The outgrowing sympathetic nerve fibers develop in the midline of the body of embryos 4-7 wpc (Wallace and Burns, 2005; Fu *et al.*, 2004) – simultaneously with the time window where the PGCs are migrating towards the developing gonads.

Not only the PGCs but also the neural progenitor cells of the ENS migrate along nerve fibers as they enter the gut (Erickson *et al.*, 2012; Burns and Douarin, 1998; Baetge and Gershon, 1989). In avian, sacral neural crest cells may use the nerve of Remark as a migration route (Burns and Douarin, 1998) and in mice, both sacral NCCs (Erickson *et al.,* 2012) and vagal NCCs (Baetge and Gershon, 1989) have been suggested to migrate on nerve fibers into the gut. In the present study the reactivity for β-III-tubulin is also noticed in the ovarian medulla and in the epithelium of the gut. β-III-tubulin is generally considered a neuron specific marker. However, a number of non-neuronal tumors as well as the mitotic spindle also stain by β-III-tubulin (Jouhilahti *et al.*, 2008). Although previous studies showed the presence of fine nervous fibers of the embryonic human gonad (Mollgard *et al.,* 2010), further studies are needed to map the pattern of this early innervation of the gonads, perhaps using electron microscopy.

In conclusion, we demonstrate for the first time the presence of OCT4-positive human PGCs in the hind gut epithelium in embryos aged 4 to 6 wpc. After leaving the gut epithelium it is suggested that chemotaxic mediators such as SCF guide the PGCs. When connections between the enteric- and the sympathetic nervous systems are established, the PGCs follow the nerve fibers of the sympathetic system towards the gonads. PGCs, which fail to exit the nerve branches at the gonadal site, continue along the sympathetic trunk ending up in other organs where they may form GCTs if not eliminated by apoptosis.

Materials and Methods

Sample collection and preparation

Samples from seven embryos and nine fetuses were obtained from either the archives of the Department of Cellular and Molecular Medicine (ICMM) at the University of Copenhagen or from legal abortions performed at the Department of Obstetrics & Gynaecology, Frederiksberg Hospital, in collaboration with the Laboratory of Reproductive Biology, Rigshospitalet, all from Copenhagen University Hospitals. Oral and written information was given and informed consent was obtained from all contributing women, according to and approved by The Regional Committee on Biomedical Research Ethics of Copenhagen and Frederiksberg (KF (01) 258206). Crown-rump lengths of the embryos and fetuses were measured and their ages were estimated in days or weeks following conception. They ranged from 5 - 103 mm crown-rump length (CRL) corresponding to 4th - 14th wpc.

Immediately after the surgical procedure the samples were dissected and fixed for 12-24 hours at 4°C in either 10% neutral buffered formalin, 4% Formol-Calcium, Lillie's or Bouin's fixatives. After fixation the samples were dehydrated in graded ethanol, cleared in xylene and paraffin embedded. Serial sections, 3-5 μm thick, were cut in transverse, sagittal or horizontal planes and placed on silanized slides. Representative sections of each series were stained with haematoxylin and eosin or with toluidine blue for orientation.

Immunohistochemistry

Sections were deparaffinized and rehydrated in xylene followed by a series of graded alcohols in accordance with established procedures. The sections were treated with a fresh 0.5% solution of hydrogen peroxide in methanol for 15 min for quenching of endogenous peroxidase and were then rinsed in TRIS buffered saline (TBS, 5 mM Tris-HCl, 146 mM NaCl, pH 7.6). Nonspecific binding was inhibited by incubation for 30 min with blocking buffer (ChemMate antibody diluent S2022, DakoCytomation, Glostrup, Denmark) at room temperature. The sections were then incubated overnight at 4 °C with primary antibodies diluted in 10% goat serum.

We used mouse monoclonal antibodies against β-III-tubulin (Abcam,

ab14545, dilution 1:15000) and against human SCF (IgG_{2b}, sc-13126 Santa Cruz Biotechnology Inc, 1:20/1:50). In the case of SCF the sections were boiled in citrate buffer, pH 6. Sections intended for staining with polyclonal rabbit–anti human CD 117 (c-Kit, A4502 DakoCytomation, 1:100/1:300) were boiled in TEG-buffer, pH 9. The polyclonal antibody against OCT4 (Abcam, Ab19857) diluted 1:250 was used without microwave oven boiling.

The primary antibodies were detected using DakoCytomation EnVision+DualLink System, Peroxidase (DAB+), code K4065, used at the manufacturers recommendation. As negative controls, sections were incubated with Rabbit IgG (X0903 DakoCytomation), Mouse IgG_{2b} (X0944 DakoCytomation) or Mouse IgG1 (X0931 DakoCytomation) instead of the primary antibody. As positive controls, sections from tissues with known occurrence of the antigens were investigated. It is well-known that mast cells express the c-Kit receptor (Hoyer *et al.,* 2005).

Double staining for β-III-tubulin and c-Kit: Prior to staining, nonspecific binding was inhibited by incubation in 0.3 % hydrogen peroxide for 15 minutes followed by 30 minutes with 0.2% casein (Sigma, C-7078) at room temperature. The sections were then incubated at 4°C overnight, with a mixture of β-III-tubulin (Abcam, ab14545, mouse, dilution 1:15000) and c-Kit (CD 117) (Dako, A4502, rabbit, dilution 1:100) in 0.2% casein, and then absorbed in human serum (known female donor). They were then incubated for 30 minutes at room temperature with Labelled Polymer –HRP anti-rabbit (Dako, EnVision™+ System/HRP K4003) followed by Tyramid Signal Amplification (Invitrogen, Molecular Probes, T20912) for 7 minutes at room temperature, subsequently 30 minutes at room temperature with DyLight 594 goat anti-mouse (Jackson ImmunoResearch, 115-515-146, dilution 1:500), and coverslipped with Dako Fluorescence Mounting Medium (Dako, S3023). The confocal images were obtained with a Carl Zeiss LSM 780, the following objectives, lasers and emission filters were used: a Plan-Neofluar 10x/0.30 M27, and a Plan-Neofluar 20x/0.4 Korr M27 objective were used. For excitation of Alexa Fluor 488, a 488 Argon laser with an emission filter 493-590 was used, and for excitation of DyLight 594 an In *Tune* laser with an emission filter 596-692 using the GaAsP spectral detector was used.

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