Human primordial germ cells migrate along nerve fibers and Schwann cells from the dorsal hind gut mesentery to the gonadal ridge

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Abstract

The aim of this study was to investigate the spatiotemporal development of autonomic nerve fibers and primordial germ cells (PGCs) along their migratory route from the dorsal mesentery to the gonadal ridges in human embryos using immunohistochemical markers and electronmicroscopy. Autonomic nerve fibers in the dorsal mesentery, the pre-aortic and para-aortic plexuses and in the gonadal ridge were stained for β III tubulin, neuron specific enolase (NSE), and glia fibrillary acidic protein (GFAP). Electron microscopy demonstrated the presence of neurofilaments and neurotubules in these nerve fibers and their intimate contact with PGCs. PGCs expressed GAGE, MAGE-A4, OCT4 and c-Kit. Serial paraffin sections showed that most PGCs were located inside bundles of autonomic nerve fibers with the majority adjacent to the most peripheral fibers (close to Schwann cells). We also show that both nerve fibers and PGCs arrive at the gonadal ridge between 29 and 33 days pc. In conclusion our data suggest that PGCs in human embryos preferentially migrates along autonomic nerve fibers from the dorsal mesentery to the developing gonad where they are delivered via a fine nerve plexus.

Key words: human germ stem cells /PGCs / immunohistochemistry and electron microscopy / migration / nerve fibers
Introduction

The seminal work of Witschi (1948) demonstrated that human primordial germ cells (PGCs) first appear among endoderm cells in the wall of the yolk sac close to allantois in 24 days post coitus (pc) old embryos and then migrate by amoeboid movement along the dorsal mesentery of the hindgut arriving at the primitive gonads at the beginning of the 5th week pc (Ding et al., 2008; Witschi, 1948). Later studies have found that the majority of the PGCs remains in the proximal end of the yolk sac, which becomes part of the wall of the hind- and midgut close to aorta as the lateral folding is completed in the beginning of the 5th week pc (Freeman, 2003). Consequently, in order to reach the gonadal anlage the PGCs only have to move from the hind- and midgut through the dorsal mesentery to the pre-aortic region in the midline of the abdominal dorsal body wall and then a little further lateral to the field where the mesonephric anlage is already formed. Thus the PGCs do not seem to migrate that far in order to reach the gonadal ridges neither in the human (Freeman, 2003) nor in the mouse (Hara et al., 2009) as Witschi (1948) originally proposed. Actually, Freeman (Freeman, 2003) pointed out that the PGCs are present already from around day 24 pc in the hindgut close to somite 16 where the gonads develop. However, the PGCs must still be transferred from the pre-aortic region and to the gonadal ridges. A number of studies have reported that human PGCs are found in the developing hindgut and in its dorsal mesentery (Jeon and Kennedy, 1973; Richardson and Lehmann, 2010) on their way to the gonads, but the mechanisms by which the PGCs are delivered to the gonadal anlage remain largely unknown. Some studies suggest that a chemotaxic receptor-ligand interaction takes place, e.g. interaction between stem cell factor (SCF) and its receptor c-Kit (Besmer et al., 1993; Fleischman, 1993; Pesce et al., 1997; Farini et al., 2007). Several extracellular components may also be important for guiding the PGCs such as glucosaminoglycans (Pereda et al., 1998; Soto-Suazo et al., 2002), fibronectin (Wylie, 1993) and collagen and tenascin C (Soto-Suazo et al., 2004). The chemokine SDF1/CXCL12 and its receptor CXCR4 regulate not only PGC migration but also survival (Molyneaux et al., 2003). Moreover, the neuropeptide PACAP was found to bind to mouse PGCs and stimulate their in vitro proliferation (Pesce et al., 1996). Further, it was recently shown that in Drosophila lipid signals cause attraction of migrating germ cells (Renault and Lehmann, 2006), a mechanism which may also
function in mammals (Richardson and Lehmann, 2010). In a histochemical study of the appearance and localization of c-Kit and SCF in human embryonic gonads and PGCs in embryos Hoyer et al. (2005) observed that PGCs were present within some autonomic nerve fiber plexuses of the dorsal mesentery and in the dorsal body wall and suggested that nerve fibers might be involved in the guidance of PGCs to the gonadal ridges (Hoyer et al., 2005).

In order to evaluate a possible migration of PGCs along autonomic nerve fibers of the hindgut the aim of the present study was to investigate (A) the appearance and distribution of the early nerve fiber plexus of the enteric nervous system with a panel of neural histochemical markers in four to eight weeks pc human embryos. In parallel, another panel of markers was tested in order to identify (B) the most suitable marker for migrating primordial germ cells. The expression of PGC and neural markers showed that most PGCs were located within bundles of autonomic nerve fibers. This observation was further substantiated by electron microscopy. Moreover, the majority of PGCs in nerve fiber bundles was located close to the most peripheral nerve fibers adjacent to Schwann cells. These observations provide strong evidence that PGCs preferently ascend from the mesentery of the hindgut to the gonadal anlage by migration along autonomic nerve fibers close to Schwann cells. We propose that these nerve fibers and/or Schwann cells may release chemo-attractants and/or transmit survival signals that may support PGC migration/proliferation.
Materials and Methods

Twelve human embryos (from 29 to 54 days post conception (pc)) and three early fetuses (8.0, 8.5 and 9.0 weeks pc) were obtained in connection with legal abortions. 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 Copenhagen and Frederiksberg Counties (KF (01) 258206). Calculation of gestational age was based on information about the last menstrual period, and measurements of crown rump and foot lengths. Embryonic and fetal age post conception, i.e. pc, was used in this study and obtained by subtracting 14 days from the gestational age informed from the gynaecological clinic. The sex of the three smallest embryos was unknown but all others were females according to a polymerase chain reaction with X-Y homologous primers on small pieces of somatic tissue (Lutterodt et al., 2009; Nakahori et al., 1991). The sex of the fetuses was determined by the gonadal morphology.

Fixation and embedding: As quickly as possible, and at least within 3 hours after abortion, the specimens were either fixed in toto for immunohistochemistry in 10% buffered formalin or in Bouin's fixative (in case of the four smallest embryos less than 37 days pc), or dissected into appropriate tissue blocks containing the gonadal-mesonephric-mesenteric complex, and fixed for 12-24 h at 4°C in one of the following fixatives: 10% buffered formalin, 4% Formol-Calcium, Lillie's AAF or Bouin's fixatives. The specimens were dehydrated with graded alcohols, cleared in xylene and embedded in paraffin wax (Merck, melting point 52°C). 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, PAS-haematoxylin and eosin, or with toluidine blue.

Electron microscopy: Some tissue blocks containing the gonadal-mesonephric-mesenteric complex intended for electron microscopy (EM) were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer adjusted to pH 7.4 for 5 - 20 hrs. Following a wash for 12 hours in the buffer the tissue blocks were post-fixed for 2 h in 2% OsO₄ in
0.1 M cacodylate buffer and stained en bloc in 4% aqueous uranyl acetate for 1 h, dehydrated in increasing concentration of ethanol, transferred to propylene oxide and embedded in Araldit. Sections, 1µm thick, were stained with toluidine blue and examined in the light microscope. Ultrathin sections were cut from selected areas and post-stained with 4% uranyl acetate and lead citrate.

**Immunohistochemistry:** Sections were de-paraffinized, rehydrated and washed in TBS (0.05 M Tris, pH 7.6, 0.15 M NaCl) with 0.01% Nonidet P-40 (TBS/Nonidet). Antigen retrieval of five of the seven antigens was performed for 10 min in a microwave oven using TEG buffer, pH 9 (see Table 1). After heat treatment the sections rested for 20 min at room temperature. In order to block endogenous peroxidase activity sections were incubated in 0.45% H₂O₂ in TBS/Nonidet for 15 min, and then in 10% normal goat serum in TBS/Nonidet for 30 min at room temperature to block non-specific binding. All sections were incubated overnight at 4°C with the following primary antibodies. Markers for (1) nerve fibers: Tubulin βIII (Tuj1), glial fibrillary acidic protein (GFAP) and neuron specific enolase (NSE), and for (2) stem cells: OCT4, GAGE, Mage-A4 and c-Kit. Details concerning primary antibodies and dilutions are shown in Table 1. For detecting mouse and rabbit primary antibodies, DakoCytomation: EnVision™ System, Peroxidase, mouse/rabbit, DAB. Code K1392, were used. For detecting goat primary antibodies Amersham Biosciences RPN 1025V diluted 1:20 in 10% donkey serum/TBS – DakoCytomation and StreptABComplex/HRP Code K 0377 – DAB, were used. Controls sections were incubated with mouse IgG3, IgG2a, irrelevant goat or rabbit antibodies, as well as subjected to omission of primary or secondary antibodies. Regarding OCT4, preincubation was performed with the corresponding peptide in the proportion 1 to 5 one 1 hour before incubation.
Results

Histochemical markers and the early developing enteric, sympathetic and sacral parasympathetic nervous system from 29 days pc to 63 days pc.

β III tubulin and NSE, showed a distinct reactivity in nerve tissues already in the 29-day-old embryo (Fig. 1A) and NSE (56 days pc: Fig. 5A). A similar but weaker reactivity and distribution was seen for GFAP (37 days pc, Fig. 1B). All control incubations were negative.

β III tubulin was strongly expressed in well-developed spinal ganglia and spinal nerves in the earliest embryos examined (29 and 33 days pc). A dense network of nerve fibers connected the sympathetic trunk with the para-aortic and aortic sympathetic nerve plexuses at 33 days pc (Fig. 1A) particularly in the region adjacent to the dorsal mesentery. At 29 days pc the enteric nervous system comprised only few weakly stained ganglionic cells and nerve fibers in the gut mesenchyme of the midgut and hindgut, and nerve fibers connecting the very immature enteric nervous system with the sympathetic aortic nerve plexuses via the dorsal mesentery were not visible at the light microscopic level (not shown). At 33 days pc the enteric nervous system of the mid- and hindgut was more developed (Fig. 1A) but nerve fibers connecting the enteric and sympathetic nervous system were scarce (Fig. 3B). At 37 days pc spinal nerves and the sympathetic trunk showed a positive staining for both β III tubulin and GFAP (Fig 1B). At this stage of development a fine, but dense network of nerve fibers extended from the enteric nervous system via the dorsal mesentery to the pre-aortic sympathetic nerve plexus (Fig. 1C). At 40 days pc nerve fibers from the sacral parasympathetic nervous system intermingled with the enteric and sympathetic plexuses (not shown).

Histochemical markers for PGCs and related nerves in the embryo

Staining for three PGC markers (OCT4, GAGE and c-Kit) were evaluated in all specimens. A distinct nuclear, cytoplasmic and cell membrane staining were detected for OCT4, GAGE and c-Kit, respectively, in PGCs located in the wall of the hindgut, the dorsal mesentery (Figs. 1C, Figs. 3, C,D, Figs. 4B,C,D, Fig. 5) and in the gonadal ridges (Fig. 2, Fig. 4, Fig. 6). Cell membranes and cytoplasm were not OCT4-positive and no other cell types or single cells but PGCs showed OCT4 reactivity (Fig. 5). Following
staining for c-Kit the PGCs expressed a strong reactivity in the cell membrane and a more faint cytoplasmic reaction (Fig. 4C). In addition, capillaries and mast cells also expressed c-Kit (Fig. 4C).

PGCs showed GAGE expression in the nucleus as well as in the cytoplasm and outlined the cells visualizing fine cytoplasmic extensions (Fig. 5C inset). The number and distribution of OCT4- and GAGE-expressing PGCs in the gonadal ridge appeared to be similar (Figs. 2 A,E, Figs. 3C,D, Figs. 4B,D). A strong β III tubulin reactivity was observed in a dense nerve fiber network in the central core of the differentiating ovary, whereas the ovarian surface epithelium appeared devoid of this reaction (Fig. 2C, D). Only a fraction of the Germ cells stained with MAGE (Fig. 2 F).

**Relationship between PGCs and nerve fibers outside the gonadal anlage.**

Immunohistochemistry: At 29 days pc the few OCT4-, c-Kit- and GAGE-positive PGCs present were neither in contact with the small number of β III tubulin-positive ganglionic cells and nerve fibers in the gut mesenchyme of the midgut and hindgut nor clearly associated with any detectable nerve fiber in the dorsal mesentery (not shown). At 33 days pc the enteric nervous system of the mid- and hindgut was more developed (Fig. 3A) and at this stage a few β III tubulin-positive nerve fibers were present in the dorsal mesentery. This is in marked contrast to the dense network of nerve fibers in the dorsal body wall, which connected the sympathetic trunk with the para-aortic and pre-aortic sympathetic plexuses at 33 days pc (Fig. 3A) particularly in the region adjacent to the dorsal mesentery (Fig. 3B). Few scattered PGCs identified by their expression of OCT4 and GAGE were seen in the mesenchyme of the midgut, whereas the dorsal mesentery showed some migrating PGCs (Figs. 3 C&D) which may be in contact with nerve fibers (compare B with D). This is, however, difficult to observe at the light microscopical level, but a few days later this can be confirmed by electronmicroscopy (Fig 3, G). In the hindgut wall and caudal dorsal mesentery an overlapping expression of the PGC markers was evident (Figs. 4A-D). Numerous migrating PGCs were present in the adrenal-gonadal-mesonephric region at this stage of development.

In 6 and 7 weeks-old embryos c-Kit-, OCT4-, GAGE- and Mage-positive PGCs were rarely seen in the hindgut itself. In the dorsal mesentery, some of the c-Kit-, OCT4-
and GAGE positive PGCs were located just beneath mesothelial cells. A large number of PGCs were in close contact with a prominent autonomic nerve plexus that stained for β III tubulin and NSE, particularly on level with the lowest thoracic columnar segment. The staining of the nerve fibers with antibodies against β III tubulin corresponded well with that seen after staining for NSE (Fig. 5A, B). In many series of serial sections individual nerve fibers of the aortic nerve plexus, which extended laterally to the gonadal and mesonephritic region, showed ‘empty areas’ or ‘holes’ (Fig. 5, A, B) which clearly corresponded to PGCs identified by expression of the markers GAGE, c-Kit, MAGE and OCT4 in neighbouring sections (Fig. 5, C-F). The cytoplasmic expression of GAGE and MAGE was detected in the very same PGCs that showed membrane staining for c-Kit and OCT expression within the nuclei. The PGCs were not randomly distributed within the autonomic nerves but were preferentially in contact with the most peripheral nerve fibers. The framed area in Figure 5C is shown in higher magnification in the inset, where two cytoplasmic protrusions of a GAGE expressing PGC appear to be in direct contact with the peripherally situated Schwann cells.

Electron microscopy: In 1 µm toluidine blue-stained araldite-embedded sections of the hind gut- midgut / dorsal mesentery-region the PGCs appeared as large often spherical cells (15 to 22 µm in diameter), with a pale cytoplasm and a rounded nucleus exhibiting a distinct nuclear membrane and a prominent nucleolus (Figs. 3 E, F). At the EM level these two PGCs showed sparse organelles (Fig. 3G). Besides small rounded mitochondria, small Golgi apparatuses, poorly developed endoplasmic reticulum and free ribosomes, lipid droplets and glycogen particles were frequent. In the dorsal mesentery, in the aorta-mesonephric area the PGCs were in intimate contact with nerve fibers and were mostly situated between the individual fibers (Fig. 3G)

Nerves and PGCs/oogonia inside the ovary

Immunohistochemistry: The staining pattern and distribution of the PGCs labeled by OCT4 or GAGE was compared to the β III tubulin expression in serial sections of the early ovary. On day 29 pc virtually no nerves were detected in the ovarian anlage. However, from day 33 pc distinct cell membrane-, cytoplasmic- and nuclear expression of c-Kit, GAGE and OCT4 were detected in the oogonia of the ovarian anlage (Fig. 4, A-
D). Interestingly, β III tubulin apparently also stained the peripheral cytoplasm of the PGCs in particular in the developing gonad. However, it is difficult to judge whether this staining could derive from tiny nerve extensions surrounding the PGCs (Fig. 4A inset). Around 56 days pc a dense nerve fiber network stained for β III tubulin was observed in the central part of the ovary (Fig. 6 A, B, C). A scant network of mesonephritic nerve fibers extended via very fine connections to an intricate network of fine dense nerve fibers in the central part of the ovary. Most of the PGCs were located adjacent to these thin nerve fibers that sometimes formed a cup around the PGCs, an “egg cup” (Fig. 6C).

Electron microscopy: After arrival to the gonadal ridges the PGCs remained in close contact to nerve fibers that extended into the gonadal tissue proper. Many oogonia were in direct contact with and virtually surrounded by nerve fibers identified by their content of neurotubules and neurofilaments (Figs. 6D&E), supporting the “egg cup” structure seen at the light microscopic level after staining for β III tubulin (Fig. 6 C).
Discussion

By using four stem cell markers for PGCs and three neural markers as well as electron microscopy we found that PGCs of human embryos were often situated adjacent to axons and their Schwann cells during their transition from the dorsal mesentery, passing through the pre-aortic region to the arrival in gonads. This supports and extends our previous observation that PGCs were in close contact with autonomic nerve fibers of the dorsal mesentery (Hoyer et al., 2005) and suggests that neurons and/or their Schwann cells might release chemo-attractants, which may be important for guiding PGCs towards and into the gonads.

The nerve supply of the developing ovary has been investigated at the light microscopic level in several mammalian species throughout the past century, recently also in the human (Anderson et al., 2002; Anesetti et al., 2001). The ultrastructure of human embryonic gonads and PGCs has previously been studied (Fujimoto et al., 1977; Fujimoto et al., 1977; Sathananthan et al., 2000). However, none of these studies mentioned the presence of nerves in connection with the PGCs, and only a few studies have focused on the very early gonadal anlage in relation to innervations. In the pig it was demonstrated that a subset of neurons of the para-aortic sympathetic ganglia migrates laterally towards the gonadal-mesonephric ridge at 21 days pc, simultaneously with the first germ cells entering the gonadal anlage (Dees et al., 2006). Although this study did not relate the route of PGC migration to the nerve growth pattern the authors mentioned the simultaneous arrival of nerves and PGCs to the gonadal ridges. This parallels the results of the present study on early human embryos, which show that both nerve fibers and PGCs arrive at the gonadal ridge between 29 and 33 days pc. The vagal neural crest cells which give rise to the enteric nervous system have been shown to enter the foregut at week 4 pc and reach the terminal hindgut at week 7 pc (Wallace and Burns, 2005). These findings thus corroborate our observations of detectable nerve fibers between days 29 and 33 pc in the aorta-gonadal-mesonephric region.

Despite important differences several features of PGC migration are the same in different organisms like flies, fishes, and mammals, e.g. proper guidance involves both attractive and repulsive cues and is mediated by protein and lipid signaling (Richardson
and Lehmann, 2010). One of the attractive cues, the stem cell factor (SCF)/c-Kit signalling pathway, is important for motility and survival of germ cells during migration towards the gonadal anlage as well as for further germ cell development in the testis and ovary (Hoyer et al., 2005; Mauduit et al., 1999; Sette et al., 2000; Pesce et al., 1993). In mice, using a PGC-specific reporter line in live embryos, it has been shown that SCF expression by somatic cells continuously surrounds PGCs throughout their migration and promotes general motility, but does not provide directional information (Gu et al., 2009). Interestingly, our previous study showed that the enteric nervous system of the mid- and hindgut expresses SCF, which might facilitate migration and survival of PGCs along the nerve fibers (Hoyer et al., 2005). In support of these arguments, certain chemokines such as stromal cell-derived-factor 1, SDF-1, are crucial for directed migration of both nerve cells (Belmadani et al., 2005) and PGCs (Molyneaux et al., 2003; Doitsidou et al., 2002).

Recently Renault and Lehmann published an article that summarizes the role of lysophosphatidic acid (LPA) as a chemo-attractant in cell migration, e.g. migration of lymphocytes, smooth muscles cells and germ cells (Renault and Lehmann, 2006). In fact, LPA-like factors are released by cultured Schwann cells (Weiner et al., 2001). Since Schwann cells differentiate simultaneously with outgrowing nerve fibers (Fu et al., 2004) it is possible that these cells may release LPA and thus play a role by attracting and guiding the PGCs. We found that the PGCs were not randomly distributed between the autonomic nerve fibers but often placed adjacent to the most peripheral fibers close to the Schwann cells. It is possible that Schwann cells may exert a chemo-attractant effect to the PGCs and/or act on cellular adhesion between the two cell types. In the mouse cholesterol seems to be mandatory for PGC migration and survival although de novo cholesterol synthesis is not mandatory for this action (Ding et al., 2008).

Germ cell specific markers like MAGE-A4 (Aubry et al., 2001) and MAGE-A1 (Gjerstorff et al., 2007) are expressed differentially in testicular germ cell tumours and in the developing testis. We found that a high number of the PGCs in the autonomic nerve fibers as well as in the young gonadal ridges express MAGE-A4, but that only a fraction of the PGCs/oogonia in the differentiating ovary expresses MAGE-A4. The migration pattern of PGCs along the nerves may explain the relatively high incidence of neural tissue related germ cell tumors (Echevarria et al., 2008) and germ cell tumors occurring
in the midline (Oosterhuis et al., 2007). It has been proposed that such tumors arise from stray PGCs as they migrate from the yolk sac along the hindgut to the mesenteries to the gonadal ridges (Teilum, 1965). Possibly these PGCs may miss the guidance towards the gonadal ridges and follow the expanding nerve system in a wrong direction.

Active cell migration has been studied in relation to formation of many organs. However, during embryogenesis many cells become displaced passively as the result of growth and folding, e.g. passive displacement of PGCs due to the lateral foldings and hindgut expansion (review: Freeman, 2003). However, the mechanisms involved in further transport of the PGCs from the hindgut to the future gonads are still uncertain but could be intimately related to the outgrowth of nerves towards the gonads. Interestingly, Pesce et al. (Pesce et al., 1996) found that the neuropeptide PACAP stimulates in vitro proliferation of mouse PGCs and proposed a novel role of neuropeptides in gonadal development. Future in vitro studies of human PGC may clarify the role of nerves and neuropeptides in PGC migration and proliferation.

In conclusion, our data suggest that PGCs in human embryos migrate along autonomic nerve fibers and Schwann cells from the dorsal mesentery to the developing gonad where they seem to be delivered via an intricate fine nerve plexus. We hypothesize that the autonomic neurons and/or their Schwann cells may release chemo-attractants and/or transmit survival signals that may support and guide PGC migration from the hindgut to the gonads.

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Reference List


**Figure Legends**

**Figure 1.** Immunohistochemical localization of β III tubulin (A) and glia fibrillary acidic protein (GFAP) (B) and OCT4 (C) in human embryos, aged 33, 37 and 53 days pc, respectively. Sagittal section through the dorsal abdominal wall showing the spinal cord (SC) with strong β III tubulin reactivity in the spinal ganglia (SG) and spinal nerves (SN) (A). Where the section crosses the midline, the aorta (AO) is seen below the floor plate (FP). The sympathetic trunk (ST) is connected with the aortic nerve plexuses (ANP) particularly in the region of the dorsal mesentery (DM). The enteric nervous system (ENS) of the mid- and hindgut is positively stained but nerve fibers connecting the enteric and sympathetic nervous system are scarce. The mesonephros (M) and the gonadal ridge (GR) possess very few nerve fibres. Transverse section through the midabdominal part of a 37 day pc embryo stained for GFAP is shown in (B). The more differentiated neurons of the ventral spinal cord (SC) extend their axons, which form the spinal nerve (SN) together with incoming fibers to the spinal ganglion (SG). The sympathetic trunk (ST) lateral to and behind the aorta (AO) is well developed and strongly stained. The insert of (B) shows a fine network of nerve fibers extending from the sympathetic nervous system via the dorsal mesentery (DM) to the enteric nervous system close to the developing gonadal ridge (GR). (C) shows a longitudinal section of the dorsal mesentery of a 53 days pc embryo, stained with OCT4. The nuclei of the PGCs are strongly stained. The majority of the PGCs is confined to the sympathetic nerves of the mesentery. The inset shows 4 PGCs confined to nerve fibers. All sections are lightly counterstained with haematoxylin. Magnification: (A & B) x 30; (C) x 75. Inset (B) x 120, (C) x 300.

**Figure 2.** Immunohistochemical localization of OCT4 (A,B), β III tubulin (C,D), GAGE (E) and MAGE (F) in serial sections from the ovary (OV) and mesonephros (M) of 63 days pc fetuses. In A and B virtually all PGCs/oogonia express nuclear reaction for OCT4. In C and D the central core of the ovary expresses a strong reactivity for β III tubulin in contrast to the more peripheral part of cortex. At higher magnification (D) the reactivity appears to be confined to a dense network of fine nerve fibers. Fibers are absent in the ovarian surface epithelium (OSE). GAGE is expressed in the PGCs/oogonia (E) and shows a similar number and distribution within the ovary as seen after OCT4 staining.
MAGE is only expressed in a few PGC/oogonia (F, inset). Magnification: (A, C, E and F) x 30; (B & D) x 75; inset (F) x 400.

**Figure 3.** Immunohistochemical localization of β III tubulin (A, B), OCT4 (C) and GAGE (D) in serial sections of the dorsal mesentery (asterisk) and the aortic nerve plexus (ANP) from a 33 days pc embryo (parallel to that shown in Fig. 1 A, but here turned upside down) and one µm toluidine blue-stained sections (E, F) and electronmicroscopic picture (G) of mesenterial-mesonephrical-gonadal area of 40 days pc embryo. The frame of A shown in higher magnification in B reveals that only a few nerves (arrows) are present in the connection between the enteric nerves (EN) and sympathetic aortic nerve plexus (ANP). Adjacent sections show OCT4 (C) and GAGE (D) expression in PGCs. E and F depict parts of the mesentery (Ms), mesonephros (M) and the gonadal ridge (GR) at day 40 pc. Two PGCs found in the mesentery (Ms) are shown in higher magnification in (F) and (G). Electron microscopy (G) demonstrates that these two PGCs are surrounded by nerve fibers (N). Magnification: (A) x 30; (B, C & D) x 120; (E) x 100; (F) x 630; (G) x 1900.

**Figure 4.** Expression of β III tubulin (A), GAGE (B), c-Kit (C) and OCT4 (D) in serial sections showing PGCs within and below the surface epithelium of the gonadal ridge of a 33 days-old embryo. The framed area in A is shown in higher magnification in the inset and depicts PGCs stained in the periphery for β III tubulin. B, C and D show that a few PGCs are present in the wall and mesentery of the hindgut (asterix). Serial sections show PGCs stained for both GAGE and OCT4 (double arrows in B and D). The c-Kit positive PGCs (arrow) in C are stained for OCT4 (arrow) in D. Beside the PGCs also the blood vessels (BV) are stained with c-Kit (C). Magnification: (A, B, C & D) x 120; inset (A) x 550.

**Figure 5.** Expression of NSE, β III tubulin, GAGE, c-Kit, MAGE and OCT4 (A-F) in serial sections of the dense pre-aortic nerve fiber plexus of a late human embryo aged 8 weeks pc showing PGCs present along nerve fibers within smaller sympathetic nerves. The aorta is labelled AO. Expression of the neuronal markers NSE and β III tubulin in A
and B shows heavily stained nerve fibers with clearly apparent unstained ‘oval holes’ (red lines in B). Adjacent serial sections stained for the PGC markers GAGE, c-Kit, Mage and OCT4 in C-F demonstrates that the ‘holes’ in the nerve fibers correspond to PGCs – see red lines in B, D and F. Note that the majority of the PGCs is in close contact with the periphery of the individual nerve fibers corresponding to contact with three perineural Schwann cells (inset in C with arrows pointing at the nuclei of the Schwann cells). At this stage of development MAGE- and GAGE positivity is present in identical serial sectioned PGCs (arrowheads in C and E). Magnification: (A - F) x 120; inset in (C) x 490.

**Figure 6.** Immunohistochemical localization of β III tubulin in low (A) and higher magnification (B&C) in an ovary (O) resting on the mesonephros (M) from an embryo, 56 days pc. Thin nerve fibers connect mesonephros (M) with a dense network of fine nerve fibers in the central part of the ovary (O) (A-C). Thin nerve fibers form a nest around the oogonia (egg cup in C). Electron microscopy from a 40 days pc ovary, showing an oogonium with cell protrusions (CP) and close contact with nerve fibers (N). The framed area in D is shown in higher magnification in E with a nerve protrusion containing neurotubules (NT) and neurofilaments (NF). Magnification: (A) x 30; (B) x 100; (C) x 400; (D) x 4000; (E) x 32.000.
Table 1

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<td>Rabbit</td>
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</tr>
</tbody>
</table>

For detecting Mouse and Rabbit primary antibodies, DakoCytomation: EnVision™ System, Peroxidase, Mouse/Rabbit, DAB. Code K1392, were used.