Primordial germ cells do not migrate along nerve fibres in marmoset monkey and mouse embryos

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Abstract

Primordial germ cells (PGCs) are the embryonic precursors of spermatozoa and eggs. In mammals, PGCs arise early in embryonic development and migrate from their tissue of specification over a significant distance to reach their destinations, the genital ridges. However, the exact mechanism of translocation is still debated. A study on human embryos demonstrated a very close spatial association between migrating PGCs and developing peripheral nerves. Thus, it was proposed that peripheral nerves act as guiding structures for migrating PGCs. The goal of the present study is to test whether the association between nerves and PGCs may be a human-specific finding or whether this represents a general strategy to guide PGCs in mammals. Therefore, we investigated embryos of different developmental stages from the mouse and a non-human primate, the marmoset monkey (*Callithrix jacchus*), covering the phase from PGC emergence to their arrival in the gonadal ridge. Embryo sections were immunohistochemically co-stained for tubulin beta-3 chain (TUBB3) to visualise neurons and Octamer-binding protein 4 (OCT4 (POU5F1)) as marker for PGCs. The distance between PGCs and the nearest detectable neuron was measured. We discovered that in all embryos analysed of both species, the majority of PGCs (>94%) was found at a minimum distance of 50 µm to the closest neuron and, more importantly, that the PGCs had reached the gonads before any TUBB3 signal could be detected in the vicinity of the gonads. In conclusion, our data indicate that PGC migration along peripheral nerves is not a general mechanism in mammals.

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Introduction

Primordial germ cells (PGCs) are the undifferentiated embryonic precursors of sperm and egg cells and are fundamental in reproduction since they are the only cells during embryonic development able to relay their genetic information to the next generation. In mice, PGCs are specified by inducing signals from cells of the proximal posterior epiblast starting at embryonic day (E) 6.0 (Lawson *et al.* 1999) and an initial population of 30–50 PGCs expressing distinctive markers has formed in the extraembryonic mesoderm by E 7.25 (Ginsburg *et al.* 1990). From there, they have to transit via the hindgut endoderm and the dorsal mesentery towards their destination, the urogenital ridges. Between E 9.5 and E 10.5 the PGCs reach the genital ridges developing laterally to the aorta where they form the embryonic gonads (McLaren 2003). In primate embryos, PGCs seem to arise in the amnion (Sasaki *et al.* 2016) and they, too, translocate to the genital ridges via the dorsal wall of the yolk sac, the hindgut endoderm and the dorsal mesentery.

The exact mechanism of this translocation from their ventral extraembryonic tissue of specification towards the dorsal body wall of the developing embryo

is still debated. Passive movement of the cells via morphogenetic changes of the surrounding tissues may play an important role in transporting the PGCs closer to their destination (Freeman 2003, Aeckerle *et al.* 2015). However, in order to move from the hind- and midgut to the gonadal ridges, active PGC migration has to be involved. Images of human PGCs published by Politzer as early as 1933 show cytoplasmic protrusions on the cells, suggesting an amoeboid migratory movement (Politzer 1933). (Molyneaux *et al.* 2001) tagged *Oct4* with GFP in genetically modified mice and thus were able to visualise and take time-lapse videos of PGCs during the stages of their migration. They showed that the PGCs exhibit active locomotion; however, this does not seem to be an intrinsically directed movement. Rather, it was proposed that the PGCs have to follow contact guidance clues or chemotactic signals in order to find the way to their site of function. Factors proposed to be involved in chemotaxis of PGCs include tumour growth factor- β (TGF β) (Godin & Wylie 1991), stromal-derived factor 1 (SDF1 or CXCL12) (Doitsidou *et al.* 2002, Bucay *et al.* 2009) and stem cell factor (SCF or kit ligand, KL) (Hoyer *et al.* 2005, Farini *et al.* 2007).

It was first suggested by Hoyer *et al.* (2005) that PGCs might be guided by nerve fibres, since they found an

association of human PGCs with autonomic nerve fibres of the dorsal mesentery in a study on c-Kit and SCF distribution in human embryos. This hypothesis was further investigated by Mollgard *et al.* (2010). In 4–8 weeks post-conception (pc) human embryos, they identified migrating PGCs and neurons and found that indeed a large proportion of PGCs were located within bundles of autonomic nerve fibres on their route from the dorsal mesentery to the gonadal ridges. They also observed by immunohistochemical marker staining that the innervation of the human gonadal ridges starts between 29 and 33 days pc, the same time that the first PGCs arrive at their destination. They concluded from their data that in human embryos the PGCs preferentially follow peripheral autonomic nerve fibres during their translocation from the dorsal mesentery to the gonads.

The aim of the present study was to investigate whether this method of PGC guidance and transition could also be observed in other mammalian species and whether it might represent an evolutionary conserved, i.e. general strategy of mammalian germ cell development. For this purpose, we investigated eight mouse embryos and eight embryos of a non-human primate, the common marmoset monkey (*Callithrix jacchus*), covering different developmental stages from the emergence of the PGCs to their arrival at the genital ridges. Tissue sections were immunohistochemically double stained and morphometrically analysed with regard to the spatial and temporal relationship of migrating PGCs to surrounding neurons.

Materials and methods

Marmoset monkey embryos and post-natal testes

All animal studies were performed in accordance with the German Animal Protection Law. The animals were obtained from the common marmoset (*Callithrix jacchus*) breeding colony of the German Primate Center (Deutsches Primatenzentrum, DPZ). Non-human primate husbandry at the German Primate Center including its institutional guidelines on housing and care of marmosets were approved by the local and regional veterinary governmental authorities (reference number 122910.3311900, PK Landkreis Göttingen). All animal work was performed by experienced veterinarians and trained staff in agreement with the requirements of the German Animal Protection Law (Deutsches Tierschutzgesetz, §7). Animal experiments to obtain the marmoset embryo material used in this study were approved by the ethics committee of the animal welfare office of the Lower Saxony State Office for Consumer Protection and Food Safety (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, LAVES), which is in charge of this approval, under licence number 425020412/0708. All parts of this study were conducted in full compliance with the German legislation requirements. Marmoset embryos of defined stages were obtained by hysterotomy as described previously (Aeckerle *et al.* 2015). All surgical procedures on the animals including anaesthesia and

analgesia were performed by a specialised and experienced veterinarian. Sections of embryos ranging from gestational day (GD) 65 to GD 90 were chosen, approximately representing the Carnegie stages 15 to 23 (Phillips 1976, Merker *et al.* 1988). Additionally, sections of three common marmoset fetuses of GD 100 and GD 117 from the tissue bank of the Platform Degenerative Diseases of the DPZ were analysed. A list of the specimens used in this study is given in Table 1.

Post-natal common marmoset testes were also taken from the tissue bank of the Platform Degenerative Diseases of the DPZ. All fixed post-natal tissue samples were pre-existing and not obtained specifically for this study, but were collected from animals that had to be killed for unrelated purposes.

Mouse embryos

Female CD1 mice were obtained from the animal facility of the European Neuroscience Institute (ENI; Göttingen, Germany), at a defined number of days after vaginal plug detection. The mice were killed by cervical dislocation and the uterus was removed for embryo collection. Embryos for this study were obtained at embryonic day (E) 8.5, E 9.5, E 10.5 and E 11.5, respectively. The list of samples can be found in Table 2.

Tissue processing and immunohistochemical double-staining

The embryos were fixed directly after retrieval in Bouin's solution for 4–24 h depending on the embryo size. Fixation was followed by several washing steps with 70% EtOH over at least 2 days. Then tissues were embedded in paraffin and sectioned at 5 µm. The embryos were positioned in order to obtain either transversal or sagittal sections. Immunohistochemical double-staining was performed in a 3-day process using the EnVision Doublestain System Kit by Dako (Code #K5361). Briefly, the slides were deparaffinised, rehydrated and an antigen retrieval was performed by heating the slides in 10 µM Na-Citrate buffer in the microwave for 10 min. Subsequently, endogenous phosphatase and peroxidase enzymes were blocked using the blocking agent provided in the kit. The first primary antibody directed against OCT4 in mice (Santa Cruz #sc-9081 (H-134), polyclonal rabbit IgG, diluted 1:150) and OCT4A in marmoset (Cell Signalling Technology #2890 (C52G3), monoclonal rabbit IgG, diluted 1:300) was incubated overnight at 4°C and stained the next day using the 3,3'-diaminobenzidine (DAB+) chromogen. After washing and blocking with an agent to prevent double staining of the same antigen, the slides were incubated with the second primary antibody against TUBB3 (Sigma-Aldrich #T8660, monoclonal mouse IgG, diluted 1:2000) over night at 4°C. All incubation steps were performed using a humidified chamber. On the third day, the second antibody-signal was visualised using Permanent Red chromogen. The slides were counter-stained for 10–15 s in Mayer's Hematoxylin Solution. Control stainings using IgG antibodies at the same protein concentration as the primary antibodies were performed in order to exclude unspecific antibody binding. Images were taken using the Leica Aperio CS2 Digital Slide Scanner and morphometrically analysed with the Aperio ImageScope software.

Table 1 Marmoset monkey (*Callithrix jacchus*) embryos/foetuses used in this study.

Embryo	GD	CRL (mm)	BPD (mm)	FROD (mm)	Sex	Sectioning plane
1	65	9.1	2.5	3.9	Female	Sagittal
2	65	9.2	2.8	4.1	Male	Sagittal
3	68	10.0	2.9	4.4	Female	Transversal
4	68	9.1	2.7	3.6	Male	Sagittal
5	72	10.5	3.0	4.7	Male	Sagittal
6	72	9.2	2.2	4.7	Female	Sagittal
7	75	N/A	N/A	N/A	Female	Sagittal
8	90	27.5	10.6	11.9	Male	Sagittal
9	100*	N/A	N/A	N/A	Male	Transversal
10	117*	N/A	N/A	N/A	Male	Transversal
11	117*	N/A	N/A	N/A	Male	Transversal

*Gestational day calculated from delivery of the previous litter.

BPD, biparietal diameter; CRL, crown-rump-length; FROD, fronto-occipital diameter; GD, gestational day; N/A, not available.

Results

PGC localisation in the common marmoset monkey (*Callithrix jacchus*)

Eight common marmoset embryos of different developmental stages (GD 65, 68, 72, 75 and 90) were analysed in this study, and a total of 853 PGCs were counted on 30 histological sections.

The PGCs were first grouped according to the anatomical compartment they were found in (Fig. 1). Looking at the total cells, approx. 3% of PGCs were detected in the epithelium of the gut, 12% either in the gut mesenchyme, the dorsal mesentery or the peri-aortic region and 14% of PGCs were attributed to the dorsal body wall. With 71% the majority of PGCs was located in the developing genital ridges, or the mesonephros and gonads in more developed embryos. Separating the sections according to the gestational day of the embryos, the proportion of PGCs that have reached the gonad expectedly increased from 13.7% on GD 65 over 47.7% on GD 72 to 93.3% on GD 90.

From each detected PGC the distance to the closest neuron was measured and the results divided into three groups: (I) Cells more than 50 µm away, (II) cells that were found between 50 and 20 µm from the nearest neuron and (III) cells with a distance of less than 20 µm.

The main finding of our examination was that for over 96% of the total detected PGCs no nerve cells could be detected within a distance of 50 µm. 1.64% of the total PGCs were found in the distance range of 50–20 µm

and only 1.75% (15 cells total) less than 20 µm away. Of these 15 cells only two were found in direct contact with a neuron (Fig. 2). Sub-dividing the sections according to gestational day does not reveal any differences in the PGCneuron distance: at least 95.3% of PGCs are found at a distance >50 µm (GD 72), 0.8–2.8% are found in the 50–20 µm distance range and only max. 3.9% of PGCs could be detected less than 20 µm from the closest neuron (GD 72).

PGCs in the mouse embryo

In the mouse, a total of 3482 PGCs were counted on 64 sections of eight embryos of consecutive embryonic days (E 8.5–E 11.5). Looking at mouse embryos provided the advantage of a more systematic investigation of PGC migration, as migration happens over a shorter time than in the marmoset. The developmental span of E 8.5–E 11.5 covers almost the entire range of PGC migration. Additionally, it allowed us to better follow the neuronal development and draw conclusions on potential interactions with PGC migration. This observation could not be made in the marmoset embryos since the neuronal development (but not the PGC translocation!) in the earliest investigated stage (GD 65) had already progressed further than that in the oldest investigated mouse embryo (E 11.5).

Again, PGCs were first classified according to their tissue location (Fig. 3). On E 8.5 98.25% of PGCs were found either in the gut epithelium or the mesenchyme surrounding the gut. On E 9.5 the majority of PGCs (84.77%) were migrating through the gut mesenchyme and the mesentery. 7.28% were found in the gut epithelium and 7.95% had already reached the location where the genital ridges started to form. By E 10.5 the genital ridges were clearly distinguishable from the surrounding tissue, and while they now harboured most of the detected PGCs (84.67%), 9.17% of PGCs were found in the gut mesenchyme, the mesentery or the region surrounding the aorta. By E 11.5 by far the most of the PGCs (>90%) had reached the developing

Table 2 Mouse embryos used in this study.

Embryo	E	CRL (mm)	Sex	Sectioning plane
1	8.5	2.4	N/A	Sagittal
2	8.5	N/A	N/A	Transversal
3	9.5	2.9	N/A	Sagittal
4	9.5	N/A	N/A	Transversal
5	10.5	4.6	N/A	Sagittal
6	10.5	N/A	N/A	Transversal
7	11.5	5.4	Female	Sagittal
8	11.5	N/A	Male	Transversal

CRL, crown-rump-length; E, embryonic day; N/A, not available.

Figure 1 PGC locations in the common marmoset monkey (*Callithrix jacchus*) embryo. Immunohistochemical double staining of OCT4A (brown) and TUBB3 (pink) (A, B, C and D). (A) Representative sagittal overview section of a GD 65 embryo. (B) Higher magnification of area of prospective gonad in (A). (C) Sagittal section of a GD 65 embryo (different than shown in A). Black arrows highlight migrating PGCs in the gut mesenchyme. (D) Developing gonad in a GD 90 embryo containing post-migratory PGCs. Bv, brain vesicle; G(l), gut (lumen); H, heart; M, mesenchyme; NI, neural lumen; Nt, neural tube; Oe, oesophagus; Pc, plexus choroideus; U, umbilical cord. The asterisks mark the developing genital ridge. Scale bar $\triangleq$ 3 mm (A), 300 μ m (B, C and D). (E) Graphical representation of tissue locations of PGCs in all investigated slides, separately analysed for each gestational day, n =number of cells available for analysis.

Figure 2 PGC distance to closest neuron in the common marmoset monkey (*Callithrix jacchus*) embryo. (A) Sagittal section of a GD 72 marmoset monkey embryo. Immunohistochemical double staining of OCT4A (brown) and TUBB3 (pink). Inlay shows an exemplary distance measurement of a migratory PGC to the closest neuron. Green arrow highlights a PGC in direct contact with a neuron. Scale bar $\triangleq$ 200 μ m. (B) Graphical representation of the distance between the observed PGCs and the respectively closest detectable neuron, separately analysed for each gestational day, n =number of cells available for analysis.

gonads. The remaining cells were detected mostly in the peri-aortic region or the mesentery. These findings reflect the PGC migration/translocation process.

Approximately 2% of total PGCs were also found at ectopic regions (regions that deviate from the 'normal' migration route) such as the neural tube.

Measuring the distance of each PGC to the closest neuron revealed results similar to those obtained in the marmoset embryos (Fig. 4): On E 8.5 only 0.88% of cells were found at a distance of less than 20 μ m from a neuron and 0.44% in the distance range of 50–20 μ m (2 and 1 cell in total, respectively). 98.68% of cells were more than 50 μ m away. This percentage even increased in the data obtained on E 9.5, where not a single PGC was detected within a 50 μ m distance of a neuron. On E 10.5

Figure 3 PGC tissue locations in the mouse embryo. (A, B, C and D) Sections of different embryonic stages immunohistochemically stained for OCT4. (A) Representative transversal section of an E 8.5 embryo. (B) Representative sagittal section of an E 9.5 embryo. (C) Representative transversal section of an E 10.5 embryo. (D) Representative transversal section of an E 11.5 embryo. A, dorsal aorta; Gl, gut lumen; M, mesenchyme; Mes, mesentery; Nt, neural tube. The asterisks mark the developing genital ridge. PGCs are highlighted and colour-coded according to the tissue they were detected in. Scale bar $\triangleq$ 90 μ m (B), 200 μ m (A, C and D). (E) Graphical representation of tissue locations of PGCs in all investigated slides, separately analysed for each embryonic day, n =number of cells available for analysis.

and E 11.5 the results remained similar, with 98.12% and 95.02% of cells detected in the $>50 \mu$ m distance category, respectively. On E 11.5 the proportion of PGCs that were found closer to a neuron increased slightly, with 2.8 and 2.9% per category, respectively.

In the mouse embryos we observed a strong increase in PGC number between E 10.5 and E 11.5 (for comparison see Fig. 3C/D), which indicates the onset of intense PGC proliferation. Clusters of several PGCs can be found outside the gonad on E 11.5, whereas only single cells were observed on the days before.

Figure 4 PGC distance to closest neuron in the mouse embryo. (A and B) Sagittal and transversal section of E11.5 mouse embryos immunohistochemically double stained for OCT4 (brown) and TUBB3 (pink). Exemplary distance measurements between representative PGCs and the respectively closest detectable neuron. Scale bar $\triangleq$ 80 μ m (A), 60 μ m (B). Asterisk marks the prospective gonad. PGCs are highlighted and colour-coded according to the tissue they were detected in. (C) Graphical representation of the distance between the observed PGCs and the respectively closest detectable neuron, separately analysed for each embryonic day, n =number of cells available for analysis.

Comparing neuronal development between mouse and marmoset embryos

Figure 5A–C exemplarily shows an E 10.5 mouse embryo double stained for TUBB3 and OCT4 as well as single stainings of the two markers. While OCT4-positive PGCs were clearly detectable in the gonad, no TUBB3 signal was visible in either the gonad or the adjacent tissue. Figure 5D–G shows the neuronal development observed in mouse embryos by TUBB3 staining: On E 8.5, neurons were clearly visible in the developing brain and started to appear in the neural tube (Fig. 5D). The intensity of these signals increased on the E 9.5 embryos (Fig. 5E). Interestingly, a clear gradient of TUBB3-positive neurons in the neural tube from cranial to caudal was observed between those two embryonic days (data not shown). On E 9.5, first ganglia

Figure 5 Neuronal development observed in the mouse embryo. (A) Representative sagittal overview section of an E 10.5 embryo immunohistochemically double stained for OCT4 (brown) and TUBB3 (pink). Scale bar $\triangleq$ 2 mm. (B and C) Higher magnification of the area highlighted in (A), immunohistochemically stained for OCT4 (B) or TUBB3 (C). Scale bar $\triangleq$ 300 μ m. (D, E, F and G) Sections of different embryonic stages immunohistochemically stained for TUBB3. Scale bar $\triangleq$ 200 μ m. (D) Representative transversal section of an E 8.5 embryo. (E) Representative transversal section of an E 9.5 embryo. (F) Representative transversal section of an E 10.5 embryo. (G) Representative transversal section of an E 11.5 embryo. A, dorsal aorta; Bv, brain vesicle; Gl, gut lumen; H, heart; L, liver; NI, neural lumen; Nt, neural tube. The asterisks mark the developing genital ridge. Pink circles highlight appearing neurons. (H) Table shows the observed TUBB3 signal intensity in different tissues at different developmental stages in the mouse embryo.

started to appear in the mesenchyme around the gut, which did not show any histological signs of smooth muscle differentiation at this stage. While the signals in the aforementioned tissues became more pronounced on E 10.5, neurons also started to appear in the region around the aorta (Fig. 5F). This was best recognisable

on the transversal embryo sections. Presence of neurons in the mesentery was not observed but cannot be excluded. Most important for this study was the finding that by E 11.5 there was still no innervation of the gonad detectable (Fig. 5C and G), although 90% of all PGCs were present in the gonad (Fig. 3E).

These findings regarding the spatio-temporal development of the peripheral nervous system in the mouse are different from the marmoset embryos, where at the earliest investigated stage (GD 65) the neuronal development had already progressed further than that in the latest mouse embryo. In the GD 65 marmoset embryos, TUBB3 staining gave a clear signal in the brain, neural tube, ganglia, ganglia of the gut, the gut epithelium and the gut mesenchyme (Fig. 1A). There was also a strong signal in the epithelia of the mesonephros. However, importantly, also in the marmoset embryos, there was no signal detectable in the gonads of even the oldest investigated embryo (GD 90, Fig. 1D), confirming the mouse data that PGCs are present in the developing gonad before any nerve fibres can be detected in the vicinity of the embryonic gonad.

Presence of neurons in the common marmoset monkey testis

In order to answer the question at which point neurons do appear in the gonad, sections of common marmoset monkey testes of different foetal and post-natal stages were stained for OCT4A or TUBB3. The results are shown in Fig. 6. At GD 100, the seminiferous tubules had already formed and the OCT4A-positive germ cells could be found enclosed within these tubules (Fig. 6A). While TUBB3 staining resulted in signals in the epithelium of the developing epididymis, no TUBB3 was detected within the actual testis. Only some light pink background staining was seen (Fig. 6B). At GD 117, TUBB3 signal could be detected in the epithelium of the epididymis as well as in the enteric ganglia in the outer (muscle) layer of the developing gut (Fig. 6C), which was also observed in the younger embryonic stages (see Fig. 1D for comparison). Additionally, at this stage nerves were detected in the forming *tunica albuginea*, but again not within the testicular parenchyma (Fig. 6D). In the neonatal and juvenile marmoset monkey testis, TUBB3 signals could be detected in the testis capsule and also sporadically within the testis itself, then often in close proximity to blood vessels forming a neurovascular pathway (Fig. 6E and F).

Discussion

The current comparative analysis of PGC translocation in the marmoset embryo extends and quantitates previous observations in the marmoset embryo by Aeckerle *et al.* (2015), which described a wide spatio-temporal (diffuse) distribution of PGCs over a large portion of the migration

Figure 6 Innervation of the common marmoset monkey (*Callithrix jacchus*) testis. Immunohistochemical staining for OCT4A (brown, A) or TUBB3 (pink, B, C, D, E and F). (A and B) Transversal section of testis and epididymis of a GD 100 marmoset foetus. (C) Transversal section of a GD 117 marmoset foetus. (D) Higher magnification of a GD 117 marmoset foetal testis. Erythrocytes present in the blood vessels unspecifically bind the antibody thereby producing a false-positive signal. (E) Section of a marmoset monkey neonatal testis. (F) Section of a marmoset monkey juvenile testis. Epi, epididymis; Gt, gut; T, testis. The asterisks exemplarily mark seminiferous tubules. Pink arrows highlight neurons. Scale bar $\hat{=}$ 50 μ m.

period. Furthermore, we included mouse embryos in this comparative analysis as a non-primate reference species. In contrast to the marmoset, in the mouse embryos, the PGCs can be followed during their translocation almost like a regular wave or homogenous cohort of cells with predictable locations at different embryonic days.

As described in the results, the onset of intense PGC proliferation in the mouse embryo can be determined histologically by the appearance of clusters of multiple PGCs around the gonad on E 11.5, where at the same location only single cells could be observed at E 10.5. At the same time the proportion of PGCs found less than 50 μ m away from the closest neuron increases from <2% on E 10.5 to approx. 5% on E 11.5. This can be explained by the appearance of neurons in the peri-aortic space close to the gonads. Importantly, our data indicate that the neurons only appear in the vicinity of the gonad when the PGCs are already present in the gonad rather than the other way round.

Neither in the mouse nor in the marmoset embryos we detected any innervation of the gonad before the PGCs arrived there, which is in contrast to the observations in human embryos. This is probably the most important finding in this study, since it clearly speaks against the hypothesis of peripheral nerves acting as evolutionary conserved guiding structures for migrating PGCs – as it has been described for humans. Published data (Hoyer *et al.* 2005, Mollgard *et al.* 2010) and those presented here rather suggest that nerve fibre-mediated guidance of PGCs is not a conserved phenomenon in mammals and may have developed only very recently in primate evolution. In this context it is important to mention a study performed by (Sasaki *et al.* 2016) on cynomolgus monkey (*Macaca fascicularis*) embryos, which belong to the group of Old-World monkeys and are therefore evolutionarily even closer related to humans than marmosets. They investigated this question as well by double staining of migratory PGCs and neurons and came to the same conclusion that no structural associations were detectable.

The question remained at which point, and if at all, neurons can be detected in the developing common marmoset monkey gonad. Since data on the innervation of the gonad is relatively scarce, we stained sections of common marmoset testes at different foetal and post-natal stages. We found that before birth, no neurons were detectable in the testis parenchyma, which is in contrast to the reports in human embryos, and that the germ cells became incorporated into the developing seminiferous tubules around GD 100 or earlier. From this point onward, the spatial separation makes any direct physical influence of neurons on germ cell development very unlikely. But even in the post-natal testes up to the post-pubertal adult, nerves within the testis parenchyma were rare.

The markers for the visualisation of PGCs (OCT4) and neurons (TUBB3) were chosen carefully. Most importantly, the same markers were used in the human embryo study by (Mollgard *et al.* 2010) as well as the study in cynomolgus monkey embryos (Sasaki *et al.* 2016), facilitating comparison between the different species. Even if it is not shown again in this manuscript, a previous study in marmoset embryos tested several potential germ cell markers and found OCT4A to reliably identify PGCs (Aeckerle *et al.* 2015). In the study on human embryos by Mollgard *et al.* (2010), TUBB3 was shown to reliably detect neurons even in the earliest developmental stages. In our study, using TUBB3 resulted in high-quality immunohistochemical stainings with very sensitive and clear signals in both investigated species. In fact, the TUBB3 protein sequence between human and marmoset TUBB3 is 99.8% (449/450 amino acids) identical (BLASTP alignment of human TUBB3 sequence (Transcript ID ENST00000315491.11) with marmoset sequence

(Transcript ID ENSCJAT00000009328.3) using *Ensembl* database (<https://www.ensembl.org/Multi/Tools/Blast?db=core>)), further substantiating the comparability of our data and the published data (Møllgard *et al.* 2010). We therefore feel confident that our selected markers and antibodies are well suited to answer the study question.

One could argue that inclusion of the PGCs that are already found in the gonads into the analysis may bias the results, since they make up a considerable percentage of total cells but are no longer migratory. However, looking at the individual developmental days in both marmoset and mouse embryos, we see no difference in the number of cells that are closely associated with neurons, independent of the PGCs' localisation. For example, on mouse embryonic day 9.5, ~92% of PGCs are still migratory outside the gonad, and not a single PGC was detected close to a neuron (see Figs 3E and 4C). On marmoset GD 65, only approx. 14% of PGCs have reached the gonads, but more than 96% of total cells were found in the >50 µm-distance category (see Figs 1E and 2B). From this, we can only draw the conclusion that our data set suitably answers the study question and that PGC migration and translocation in the marmoset and mouse embryo are not dependent on peripheral nerve guidance to reach the gonad.

We are aware of the limitations of histological sections as they only allow 2D analysis. A closer association between PGCs and nerve cells in the third dimension cannot be completely excluded. In order to minimise this problem, consecutive sections of the same embryos were analysed to obtain a better spatial resolution. However, to analyse in detail and finally prove the spatial relationships between peripheral nerves and PGCs it would be favourable to have a 3D representation of the tissues of interest.

Using this visualisation method, the possibility that the TUBB3 signal intensity lies under the detection threshold, and present neurons might therefore not be visible, can also not be excluded. However, we think this is unlikely since, again, the findings in the human embryos were obtained by the same method and Møllgard *et al.* describe clearly detectable nerve fibres (Møllgard *et al.* 2010).

In summary, no spatial association between PGC migration and nerve fibres could be observed, neither in mice nor in two non-human primate species. This finding falsifies our hypothesis that PGC guidance by developing peripheral nerves is a conserved mechanism in mammalian embryonic development. When we compare our findings to those observed in the human embryos, we conclude that the observations by Møllgard *et al.* – other than representing a general mammalian strategy – rather reflect a species-specific trait of human PGC development.

Declaration of interest

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

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Author contribution statement

E W contributed to conduction of experiments and analysis of results, graphical representation of data, writing of manuscript. M S helped in conduction of experiments and analysis of results and in final approval of manuscript. R B contributed to study design, financial support, correction and approval of manuscript.

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