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The problem of the origin of primordial germ cells (PGCs) in vertebrates: historical review and a possible solution

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ABSTRACT A concise review of the articles about the origin of primordial germ cells (PGCs) in vertebrates is provided. Differences among various taxa concerning the origin of PGCs, not easily understandable on the base of traditional knowledge, are pointed out. All those differences can be explained taking into consideration the recent "theory of the endoderm as secondary layer". That theory allows us to understand that those differences are only apparent, being related to modifications of stages of the consequent embryogeny, overall, to a different amount of yolk in the egg. Eggs very rich in yolk became meroblastic, and the portion of primordial ectomesenchyme destined to give rise to a part of the mesoderm and the PGCs separates early from the part destined to give rise to the rest of the mesoderm and to the digestive endoderm in order to form the vitelline hypoblast lamina. To this lamina, in contrast to the traditional interpretation, a mesodermal, not endodermal, origin must be attributed. With the misunderstanding regarding the origin of this lamina clarified, all the differences about the origin of PGCs disappears. Furthermore, in taxa where PGCs were considered to be of endodermal origin, they too have a mesodermal origin. Considering that a mesodermal origin of PGCs has been demonstrated in all sponges and cnidarians, as well, a unique, mesodermal origin of germinal cells in all pluricellular animals results.

KEY WORDS: *vertebrate, primordial germ cell origin*

Introduction

The origin and the migration of Primordial Germ Cells (PGCs) in vertebrates is an interesting, unsolved, problem. The gametes mature in the gonads but arise from PGCs, which appear in various sites, more or less far away from the area in which the gonadal anlagen will be formed, and reach their destination through blood or active migration. According to the literature, in some cases the PGCs arise from the mesoderm, in others from the endoderm or from the ectoderm; and in some cases they are even said to arise from extraembryonic areas. Even within the same class, they often seem to have different origins.

This situation is, until now, difficult to understand, and there is no interpretation from a phylogenetic point of view. Since the germinal cell surely became specialized in very primitive ancestors, it is difficult to suppose a different origin of the PGCs within the same phylum or the same class.

In many cases it might appear more appropriate to use the words ectoblast, mesoblast and endoblast, but in this paper, when a precise distinction is not necessary, we will use the words ectoderm, mesoderm and endoderm, as other authors do, to define the germ layers.

Historical review

In the following historical excursus we start with the taxa showing situations which appear more primitive regarding the characteristics of the eggs and the first stages of embryogenesis.

For many years (Chiquoine, 1954), PGCs have been recognized as being larger than the surrounding cells, as possessing a prominent nucleolus, more darkly staining nuclear and plasma membrane and, overall, as having high alkaline phosphatase activity. Precursor cells of the PGCs, before showing alkaline phosphatase positivity, are recognized by the expression of the specific markers *Dazl* and *Vasa* (Yoon *et al*., 1997; Bachvarova, 2009), or the transcriptional repressor *Blmp1* detected in mice by Ohinata *al*. (2005).

Abbreviations used in this paper: PGC, primordial germ cell.

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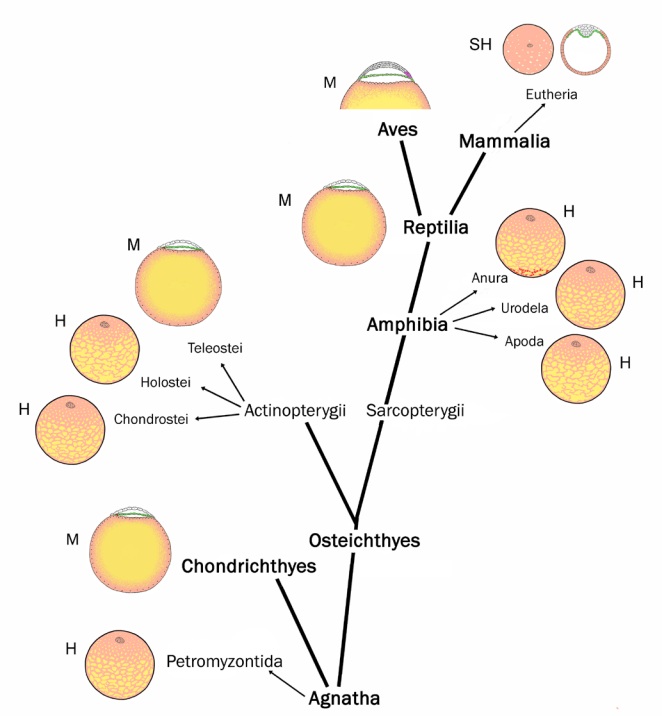

Fig. 1. Schematic, simplified, phylogenetic tree of the Vertebrata. *Only the eggs of the taxa taken into consideration in this paper are shown. For each taxon the type of egg produced or, for the meroblastic or secondarily holoblastic eggs, the early blastula is shown. The dimensional proportions are not respected. H, holoblastic; M, meroblastic; SH, secondarily holoblastic.*

To compare the events of the embryogeny, a normalization of its steps in the various taxa would be necessary, but unfortunately this is not possible and therefore we can take into consideration only accounts provided by the literature.

In Fig. 1 a schematic, simplified, phylogenetic tree is shown indicating the taxa taken into consideration in this paper.

Origin of PGCs in vertebrates producing mesolecithal, holoblastic eggs

Origin of PGCs in Amphibia

PGCs in anurans are recognizable earlier than in other vertebrate taxa by the presence of the "germinal plasm" (Fig. 2A). In these animals (having mesolecithal, holoblastic eggs) it is universally accepted that the germ cells have endodermal origin. They arise from the endodermal moiety of the egg in the vicinity of the vegetal pole. Nieuwkoop & Sutasurya (1979, page 82) wrote: "As a consequence of the pregastrulation movements, the blastomeres containing germinal plasm are displaced together with the endodermal cell towards the centre of the vegetal yolk mass and sometimes even as far as the floor of the blastocoels (Figs. 2B,C). Subsequently the PGCs are displaced by the gastrulation movements and are ultimately found among the vegetal blastomeres in the caudal portion of the embryo". When the coelom is formed, PGCs migrate through the dorsal mesentery and reach the genital ridges as active or passive travellers.

According to some authors, structures that can be interpreted as germinal plasm should be present also in urodeles, but in these animals they are less evident than in anurans and have a different functional cycle (Smith *et al.,* 1983).

Authors have expressed various opinions about the origin of PGCs in urodeles (having mesolecithal holoblastic eggs). According to many they have mesodermal origin and appear in the lateral plate mesoderm (Fig. 3) (Smith, 1964; Ikenishi & Nieuwkoop, 1978; Nieuwkoop & Sutasurya, 1979; Smith *et al.,* 1983; Johnson *et. al.*, 2003). According to others, they arise from the endoderm and migrate into the mesoderm at a relative advanced stage of embryogenesis (e.g. Blackler, 1966).

Maufroid & Capuron (1972) found that in the early neurula the PGCs are located in the neighbourhood of the blastopore from where they are displaced anteriorly along with the lateral plate mesoderm. Subsequently PGCs migrate dorsally towards the gonadal anlagen.

Experimental studies yielded contrasting results about the possibility of the ectoderm to give rise to PGCs (Smith, 1964).

When comparing the formation of PGCs in anurans and in urodeles, it must be stressed that: a) in anurans those cells originate from the endodermal moiety of the egg whereas in urodeles they arise from the animal moiety (particularly the presumptive lateral plate mesoderm); and b) in anurans the nature of the PGCs appears predetermined by the presence of the germinal plasm; in urodeles the germinal plasm appears only at a late stage of the development when the cellular differentiation has already begun. This suggests that in urodeles the germinal plasm cannot play the role of germ cell determinant (Ikenishi & Nieuwkoop, 1978), and

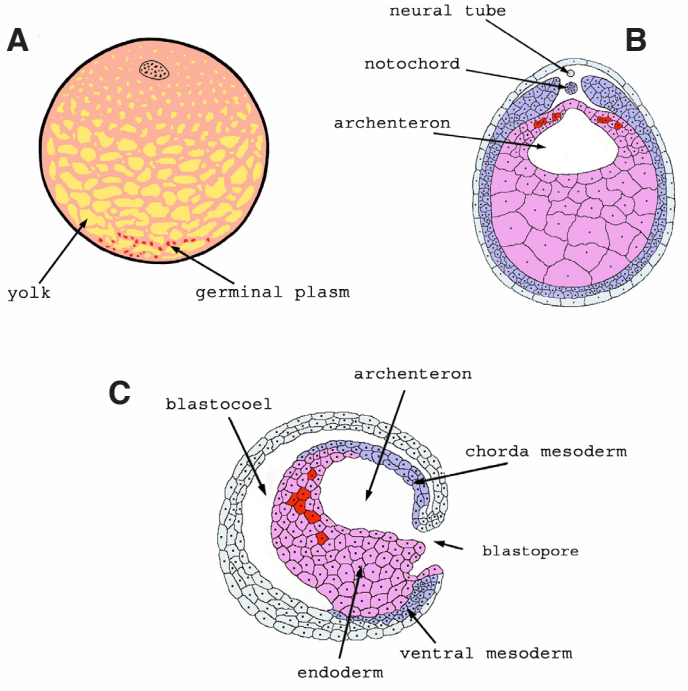

Fig. 2. Appearance of PGCs in the anuran embryo. (A) *Uncleaved fertilised anuran egg; the germinal plasm, in red, in form of subcortical patches, is still localised near the vegetal pole.* **(B,C)** *The blastomeres containing the germinal plasm (in red) intermingled with endodermal blastomeres rich in yolk, are displaced dorsally towards the floor of the blastocoel. Light-blue, ectoderm; blue, mesoderm; violet, digestive endoderm; red, germinal plasm in* **(A)**, *PGCs in* **(B)**.

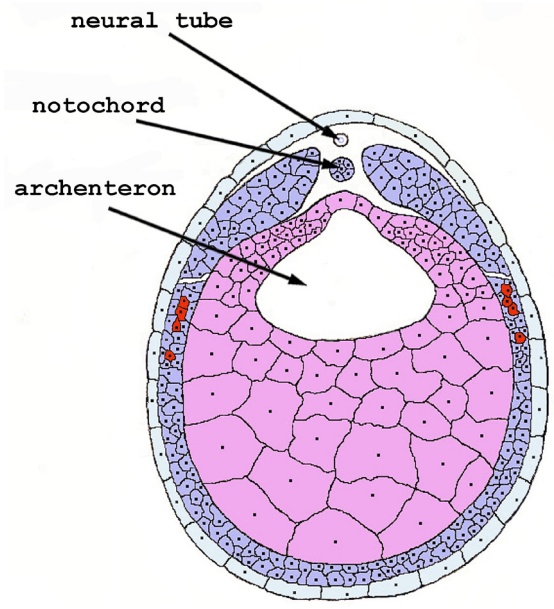

Fig. 3. Appearance of the PGCs in urodelan embryo. *PGCs in urodelan embryo (in red) become recognizable in the lateral plate mesoderm. Light-blue, ectoderm; blue, mesoderm; violet, digestive endoderm; red, PGCs.*

that the PGCs in those animals do not develop from predetermined elements but according to Nieuwkoop & Sutasurya (1979) "develop epigenetically from totipotent cells of the animal moiety under the very early inductive influence of the ventral yolk endoderm".

The data regarding Caeciliidae (Apoda) are very old. In these amphibians (very rich in yolk but still holoblastic eggs) Marcus (1938) identified PGCs in the endoderm in the vicinity of the blastopore; at later stages of development he found them in the dorsal mesentery from which they move forward to the genital ridges. It has not been demonstrated if they reach the dorsal mesentery from the dorsal endoderm or passing through the lateral plate mesoderm.

Origin of PGCs in Petromyzontida

In lampreys (mesolecithal, holoblastic eggs) Okkelberg (1921) found PGCs in the lateral plate mesoderm. Their migration route resembles that of urodele amphibians (Okkelberg, 1921; Nieuwkoop & Sutasurya, 1979).

The Myxinoida have telolecithal meroblastic eggs but we have no precise information about the origin of PGCs.

Origin of PGCs in Osteichthyes producing holoblastic eggs

Some investigated Osteichthyes have holoblastic eggs more or less rich in yolk. Both in the Chondrostei and the Holostei PGCs may be first found in the gut endoderm and in the adjacent lateral plate mesoderm (*Amia* and *Polypterus)* (Allen, 1911; De Smet, 1970), or just in the gut endoderm (*Lepisosteus* and *Acipenser*) (Allen, 1911; Maschkowzeff, 1934). In all these taxa PGCs are therefore supposed to have endodermal origin and to migrate towards the lateral plate mesoderm, and then to the dorsal midline and to the gonadal anlagen (Allen, 1911; Nieuwkoop & Sutasurya, 1979).

Origin of PGCs in Chondrichthyes, some Osteichthyes (the Teleostei) and amniotes producing telolecithal meroblastic (or secondarily oligolecithal holoblastic) eggs

Chondrichthyes, some Osteichthyes (the Teleostei) and many amniotes have telolecithal meroblastic eggs. Theria have secondarily oligolecithal holoblastic eggs. In all those cases, before discussing the origin of PGCs it is necessary to refer to the lamina which develops very early beneath the blastodisc (Fig. 4A-C), and beneath the embryonic node in therian mammals (Fig. 4D) and which later participates in the formation of the yolk sac wall. This lamina is traditionally considered as an extraembryonic structure of endodermal origin and is variously named: hypoblast, primary endoderm, extraembryonic endoderm, primary hypoblast, visceral endoderm (in the mammals) or vitelline hypoblast, etc. To avoid confusion we will call it the "vitelline hypoblast lamina" independently from the name used by the various authors (whom we previously indicated in the text).

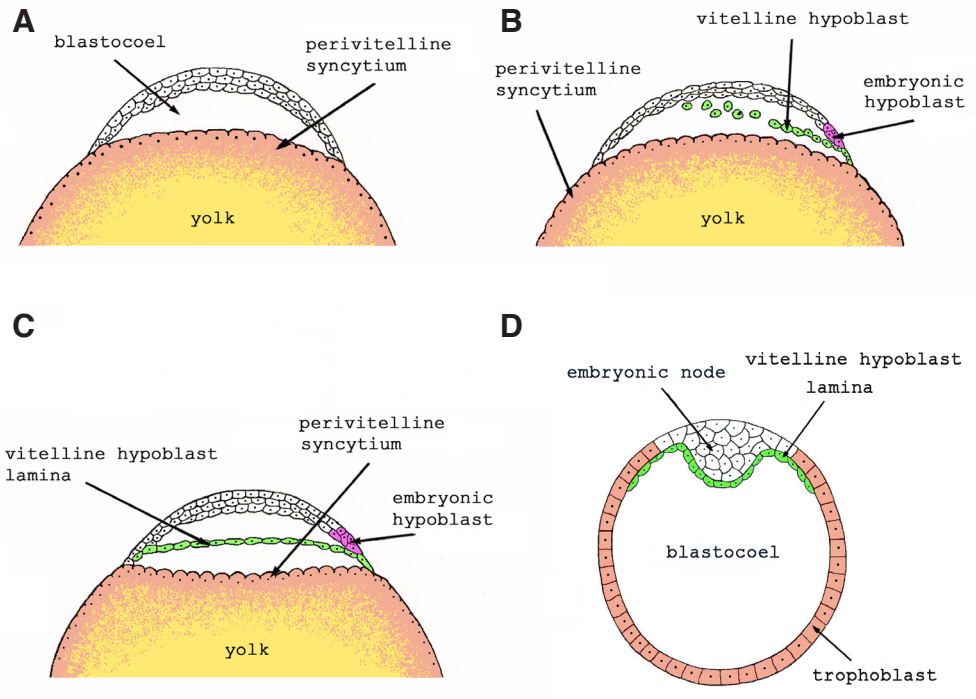

Fig. 4. Early embryonic stages of Aves and Theria. (A-C) *Avian blastula:* **(A)** *Under the blastodisc the subgerminal cavity (blastocoel) is formed. The yolk is enveloped by an ooplasmic layer (the perivitelline syncytium),* **(B)** *The vitelline hypoblast lamina (in green) settles,* **(C)** *The vitelline hypoblast lamina settled and subdivided the blastocoel into an upper and a lower portion. The embryonic hypoblast (in violet) will give rise to the intestinal epithelium.* **(D)** *Therian blastocyst. The vitelline hypoblast lamina adheres to the embryonic node. Green, vitelline hypoblast lamina; pink, perivitelline syncytium; violet, digestive endoderm; yellow, yolk.*

Origin of the PGCs in Chondrichthyes

In Chondrichthyes PGCs are first recognizable in the "dorsolateral endoderm" (= vitelline hypoblast lamina) and in the adjacent lateral plate mesoderm; some PGCs are also recognizable in the overlying ectoderm. In older embryos they are recognizable in the splanchnic mesoderm or between it and the gut epithelium (Nieuwkoop & Sutasurya, 1979).

Origin of the PGCs in Teleostei

In teleosts the origin of the germ cells is poorly known and it has been traced at different stages of embryogenesis and different layers in different fish. They may appear in the extraembryonic "yolk sac endoderm" (deriving from the vitelline hypoblast lamina) in association with the perivitelline syncytium (Johnston, 1951; Oppenheimer, 1959; Depêche & Billard, 1994), or they may segregate only from the latter structure (Johnston, 1951). However, Gamo (1961) first found PGCs in the unsegregated mesendoderm and occasionally in the ectoderm at an early gastrula stage, but after the segregation of mesoderm and endoderm, he found PGCs in all three germ layers. Pala (1970), at an early gastrulation stage, found PGCs among the "deep cells of the embryonic node" (i.e. in the vitelline hypoblast lamina) in the caudal half of the blastodisc (Fig. 5); later in the mesoderm. Pala's statements have been confirmed by more recent studies. In zebrafish, at 4-hr post-fertilization precursor cells are detectable in the lower part of the blastoderm close to the yolk syncytial layer and very few in the upper part of the blastoderm; at 8-hr post-fertilization they are detected in the hypoblast. Later, at the 1-6 somite stages, PGCs are located between the lateral mesoderm and the yolk syncytial layer (Weidinger *et al.*, 1999; Nagai *et al.*, 2001).

After their differentiation, PGCs move through the splanchnic mesoderm towards the dorsal mesentery and reach the genital ridges.

Origin of PGCs in Reptilia

In reptiles PGCs are first recognizable in the "primary hypoblast" (= the vitelline hypoblast lamina)(Fig. 6A,B) before the mesoderm settles in the "germinal crescent" which is an extraembryonic area of the blastoderm recognizable at the boundary between the area pellucida and the area opaca.

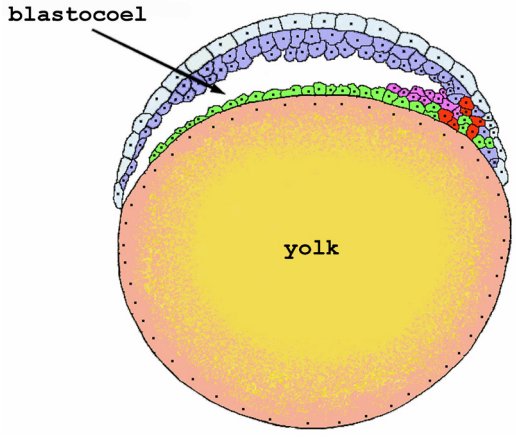

Fig. 5. Appearance of PGCs in teleostean embryo. *At an early gastrulation stage, PGCs (in red) are recognizable among the "deep cells of the embryonic node" (i.e. in the vitelline hypoblast lamina) in the caudal half of the blastodisc; later in the mesoderm. light-blue, ectoderm; blue, mesoderm; green, vitelline hypoblast lamina; violet, digestive endoderm; pink, perivitelline syncytium; yellow, yolk; red, PGCs.*

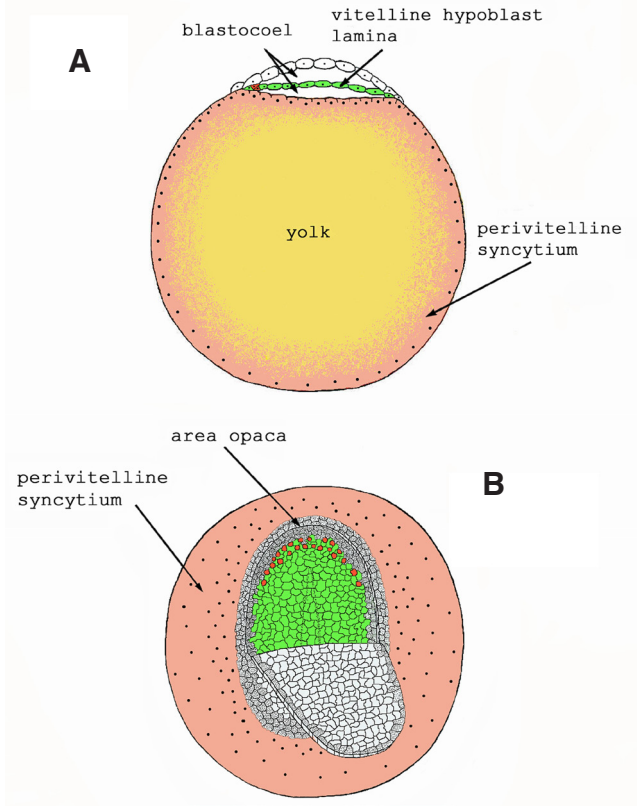

Fig. 6. Appearance of PGCs in early reptilian embryo having germinal crescent in an anterior position. (A) *In sagittal section. PGCs (in red) are recognizable in the vitelline hypoblast lamina at the boundary between the area pellucida and the area opaca,* **(B)** *Idem in polar vision. Nuclei are drawn only in the perivitelline syncytium. Light-blue, ectoderm; green, vitelline hypoblast lamina; pink, perivitelline syncytium; yellow, yolk; red, PGCs.*

This area is anterior in some taxa, posterior in others, or localized around the entire blastodisc in others. Different types of localization of this area may be present in the same taxonomic group (e.g. in Sauria and in *Sphenodon*) and therefore there is not a precise correlation between the types of localization and the taxonomic group (Nieuwkoop & Sutasurya, 1979; Hubert, 1985; Johnson *et al.,* 2003).

According to Hubert (1976), PGCs are recognized also in the definitive endoderm, and he stated (Hubert, 1985) that in some reptiles (Gekkonidae, Lacertidae and Iguanidae) those cells originate within the epiblast and assume a posterior location in the mesoderm adjacent to the cloaca.

In Chelonia, according to Bachvarova *et al.* (2009), cells that may be interpreted as PGCs, or their precursors, are first detected in the blastoporal plate, i.e. a region of nascent mesoderm.

When differentiated in a posterior germinal crescent, PGCs reach the gonadal anlagen by interstitial migration through the splanchnic mesoderm and the dorsal mesentery; when differentiated in an anterior germinal crescent, or around the entire blastodisc (*Anguis*), they reach the gonadal anlagen by vascular transfer (Hubert, 1969, 1976; Nieuwkoop & Sutasurya, 1979).

Origin of PGCs in Aves

The bird embryos have an anterior germinal crescent, more or less extended around the blastodisc, and PGCs, traditionally considered of endodermal origin, are first found very early in the "primary hypoblast" (= vitelline hypoblast lamina) of this "extraembryonic area" (Fig.7).

Probably PGCs derive from epiblast cells that migrate from the ventral surface of the area pellucida into the above mentioned extraembryonic lamina (Eyal-Giladi *et al*., 1981; Johnson *et al*., 2003). Eyal-Giladi *et al.* (1981) suggested that 95% of PGCs originate from epiblast and 5% from "hypoblast" (= vitelline hypoblast lamina). PGCs reach the gonadal anlagen by vascular transfer (Nieuwkoop & Sutasurya, 1979).

According to some authors (Johnson *et al*., 2003), in aves PGCs are predetermined by maternally inherited germ plasm, but others think that they are induced at a later stage of development because in avian PGCs germ plasm is not recognizable (Eyal-Giladi *et al*., 1976).

Origin of PGCs in therian mammals

In Theria, which have secondarily oligolecithal holoblastic eggs, the data do not seem to be concordant.

According to most authors, in 8 day mouse embryos PGCs are first recognizable in the extraembryonic "yolk sac endoderm" (deriving from the vitelline hypoblast lamina), and also according to Fujimoto *et al.* (1977) in the allantoic endoderm. Slightly later they are present in the hind gut, but they resemble mesodermal cells more closely than endodermal ones. (Clark & Eddy, 1975).

TABLE 1

VARIOUS TAXA, THEIR EGG TYPE, AND EMBRYONIC LAYER (OR STRUCTURE) WHERE PGCs (OR THEIR PRECURSORS) ARE FIRST RECOGNIZABLE

Taxon	Egg type	PGCs first recognisable in
Petromyzontida	holoblastic	lateral plate mesoderm
Anurans	holoblastic	endoderm
Urodeles	holoblastic	lateral plate mesoderm
Caeciliidae	holoblastic	endoderm (in the vicinity of the blastopore)
Some Osteichthyes (*Amia*, *Polypterus*, *Lepisosteus*)	holoblastic	- lateral plate mesoderm - gut endoderm
Other Osteichthyes	meroblastic	- ectoderm - unsegregated mesoderm - "primary endoderm" (= vitelline hypoblast) - yolk sac "endoderm" (= vitelline hypoblast) - perivitelline syncytium - vitelline hypoblast (precursors in the lower part of the blastoderm)
Chondrichthyes	meroblastic	- "dorso-lateral endoderm" (= vitelline hypoblast) - lateral plate mesoderm - epiblast
Reptiles	meroblastic	- "primary hypoblast" (= vitelline hypoblast) in the extraembryonic germinal crescent area - definitive endoderm - blastoporal region of nascent mesoderm - epiblast
Aves	meroblastic	- "primary hypoblast" (= vitelline hypoblast) in the extraembryonic germinal crescent area - epiblast
Mammals	Meroblastic or secondarily holoblastic	- extraembryonic yolk sac "endoderm" (=vitelline hypoblast lamina) - extraembryonic yolk sac "endoderm" (=vitelline hypoblast lamina) and in the allantoic endoderm - yolk sac mesoderm or "endoderm" - posterior primitive streak area in the region of the primitive endoderm fated to become the hindgut. - extra-embryonic mesoderm just posterior to the primitive streak adjacent to the base of the allantois (precursors in epiblast cells) - epiblast

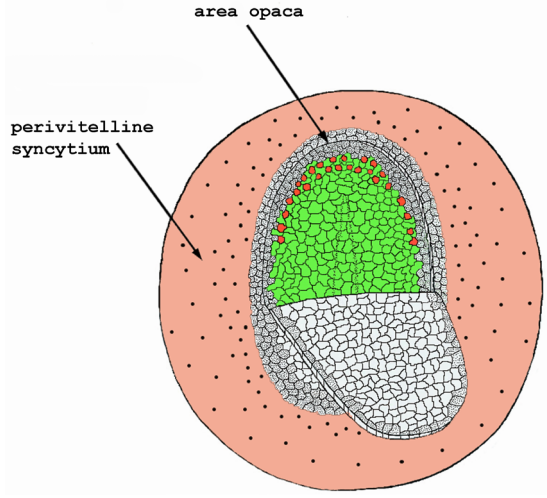

Fig. 7. Appearance of PGCs in early avian embryo. *In an avian blastula in polar vision PGCs (in red) are recognizable in the vitelline hypoblast lamina at the boundary between the area pellucida and the area opaca. Nuclei are drawn only in the perivitelline syncytium. Light-blue, ectoderm; green, vitelline hypoblast lamina; pink, perivitelline syncytium; red, PGCs.*

Chiquoine (1954) suggested that in 8 day mouse embryos PGCs originate from "yolk sac splanchnic mesoderm or endoderm" and perhaps, as later confirmed by Anderson *et al.*(2000) and Runyan (2006), from the caudal portion of the primitive streak (Fig. 8A).

Gardner & Rossant (1976) and Falconer & Avery (1978) maintained that PGCs originate from cells of the primary epiblast of the embryonic node from which both PGCs and somatic cells later derive.

In mice, around 7.25-7.5 days post coitum, PGCs are seen at the base of the allantois (more precisely in the extra-embryonic mesoderm) (Saitou *et al*., 2002), and, according to Ozdzenski (1967), also in the caudal end of the primitive streak (Fig. 8B); Snow & Monk (1983) agreed with this statement but also suggested that PGCs have an epiblastic origin.

According to more recent statements, PGCs become identifiable as a cluster of cells in the extraembryonic mesoderm at the base of the allantois (Fig. 8B), but their precursors reside in a founder population in the epiblast of the gastrulating embryo (adjacent to the extraembryonic ectoderm). These precursors give rise also to extraembryonic mesodermal lineages (Ginsburg *et al*., 1990; Lawson & Hage, 1994; Saga *et al*., 1996; Koshimizu *et al.,* 1996; Saitou *et al*., 2002; Johnson *et al*., 2003).

Eddy *et al.* (1981) and Eddy & Hahnel (1983) suggested that mammalian PGCs arise from stem cells not yet committed to other developmental fates; Ying *et al.* (2001) demonstrated, in culture, that signals from extraembyonic ectoderm induce epiblast cells to give rise to PGCs without the intervention of the visceral endoderm.

Ohinata *et al.* (2005) noted that within a cluster of epiblast cells a few cells (about six) activate the expression of *Blimp1*, a marker of lineage-restricted precursors, at pre-gastrula stages (6.0-6.5 days post coitum in mice); these cells move to the posterior primitive streak and allantois where, at 7.25 days, they become specified by the expression of alkaline phosphatase (Ohinata *et al*., 2005).

PGCs then migrate along the endoderm and the mesentery of the hind gut and colonize the genital ridges (the future gonads) (Lawson & Hage, 1994; Saitou *et al.*, 2002; Ohinata *et al.*, 2005).

Synopsis

In Table 1 the data mentioned above are summarized. The taxa, their type of egg, and the embryonic layer or the structure (embryonic or extraembryonic) where PGCs are first recognizable are indicated (as previously mentioned, a normalization of the steps of the embryogeny would be necessary for more precise comparison).

Problems and aim of the paper

It is evident that there are various unsolved problems about the origin of PGCs, some of which we intend to stress:

Why within the same class do PGCs become evident in structures of different origin (in some cases of mesodermal origin, in others of endodermal origin and in some cases, in very early blastulae, of epiblastic origin)?

Considering that PGCs are essential for the survival and continuation of the species, and are phylogenetically ancient, why are they recognizable before the gonads develop, arising outside the gonad anlagen and in some taxa even in extraembryonic regions?

In some embryos developing from telolecithal meroblastic eggs, PGCs appear in the caudal portion of the primitive streak through which the mesoderm that will give rise to the gonads settles. In spite of this, PGCs do not reach the gonad region directly, first going to extraembryonic areas thus initially departing from their destination. How do we explain this surely uneconomic behaviour?

The anurans excluded, it is not clear if PGCs are predetermined very early, or if they are determined epigenetically (and therefore later), or if they belong to a lineage of cells that retain the primitive capacity to give rise to different types of cells.

The aim of this paper is not to understand whether PGCs are predetermined or determined epigenetically (question No. 4), but to establish from where they derive.

Here we take the opportunity to stress that an eventual epigenetic determination does not explain why those cells arise outside the gonad anlagen and in many cases even in extraembryonic regions from which they have to migrate to reach their destination.

Whichever the mechanism of determination may be, it seems evident that the time of determination of PGCs precedes that of their differentiation, i.e. the appearance of the characters allowing us to recognize them (Eddy & Hahnel, 1983; Koshimizu *et al.*, 1996). This is very evident in anurans since the germinal plasm is already identifiable in the egg, and since during the egg cleavage some blastomeres, those with germinal plasm, destined to give rise to the germ cells are recognizable.

In other vertebrates the differentiation of PGCs occurs later, when the embryogenesis is more advanced, but also in these cases their destiny seems to be in some way fixed before they differentiate. Indeed in mice PGCs appear in 8-day embryos, but if a piece of the caudal portion of the primitive streak of 7-7.5 days embryos is isolated, after 24-48 hours PGCs appear (Ozdzenski, 1967; Eddy & Hahnel, 1983). This means that in the portion of embryo experimentally isolated before PGCs differentiate, cells already somehow destined to become later PGCs are present. Eddy & Hahnel (1983, pages 48-49) wrote: "The number and somewhat scattered location of appearance of alkaline-phosphatase positive PGCs suggest that the germ cell line might be established earlier, that PGCs are carried into these areas by morphogenetic movements and that expression of alkaline phosphatase activity occurs secondarily to the establishment of the germ line (Clark & Eddy, 1975; Eddy *et al.,* 1981)". This has been confirmed molecularly (Koshimizu *et al.,* 1996).

As regards the origin of PGCs, if one refers to very early stages of development, it is obvious that these cells arise from the epiblast of the early blastula (Gardner & Rossant, 1976; Falconer & Avery, 1978; Saga *et al.,* 1996; Koshimizu *et al.,* 1996), but this epiblastic origin has no phylogenetic meaning since all the embryonic structures arise from the epiblast if we refer to a very early blastula. On the contrary, an evaluation is possible when the subsequent stages of the embryogenetic process, showing variations among different taxa, are compared.

In the various vertebrate taxa PGCs become evident in some cases in regions having the same origin and in other cases in re-

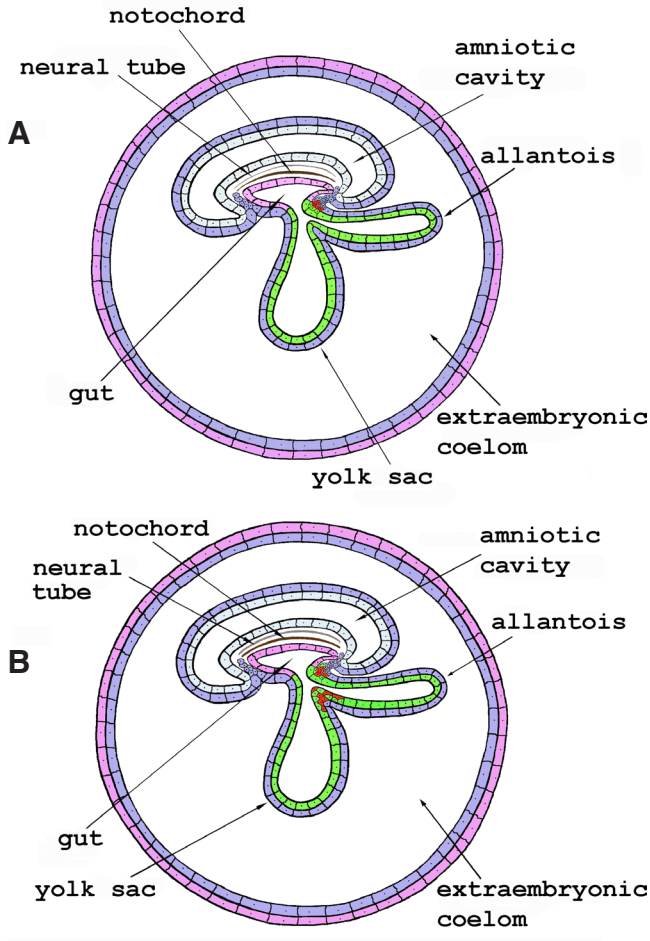

Fig. 8. Appearance of PGCs in mammals producing secondarily oligolecithal holoblastic eggs (Metatheria and Eutheria). (A) *PGCs (in red) may originate at the base of the allantois from the "yolk sac splanchnic mesoderm or endoderm", and perhaps from the caudal portion of the primitive streak, or* **(B)** *as a cluster of cells in the extra-embryonic mesoderm at the base of the allantois and in the caudal end of the primitive streak. Light-blue, ectoderm; grey, neural tube; blue, mesoderm; brown, notochord; green, vitelline hypoblast lamina; violet, endoderm; red, PGCs.*

gions that seem to be of different origin (as the embryonic layer is concerned). It is thus necessary to ascertain if, during vertebrate evolution, in some cases the area of origin of PGCs has really changed or if the change is only an appearance.

In conclusion, in our opinion, to solve the problems related to the origin of vertebrate PGCs it is necessary not to limit oneself to note the region they appear, but to understand the origin of those regions, i.e. their morphological identity since cellular movements start, and organize them.

Recent statements on vertebrate embryogenesis

In our opinion, in order to solve the previously mentioned problems about the origin of PGCs, it is necessary to keep in mind some recent statements on vertebrate embryogenesis. In particular it is necessary to keep in mind that the characteristics allowing us to recognize PGCs may appear at different times in the various vertebrate taxa. Also, since in many cases PGCs appear in the vitelline hypoblast lamina, or structures derived from it, it is first necessary to understand the meaning of this structure which Pilato discussed in previous papers (Pilato 1994, 2003, 2007).

To discuss the former problem a precise normalization of the steps of embryogeny would be necessary, but even in the absence of this normalization, differences are evident between the embryogeny of taxa producing holoblastic eggs and those producing meroblastic eggs (in particular about the vitelline hypoblast lamina which forms only in the latter case). We will discuss this problem later, since it is first necessary to clarify the meaning of the vitelline hypoblast lamina.

That lamina forms early, before gastrulation, only in embryos developing from telolecithal meroblastic eggs, and in the secondarily holoblastic eggs of therian mammals. It forms between the blastoderm and the perivitelline syncytium (Fig. 4B-C) and in therian mammals adheres to the embryonic node (Fig. 4D). That lamina is traditionally considered an extraembryonic endodermal structure, and this meaning is reflected in the various names still attributed to it (e.g. primary endoderm, visceral endoderm, embryonic endoderm, and others). However, previous research (Pilato, 1994, 2003, 2007), lead us to believe that this is a misunderstanding, and to attribute to that lamina a different meaning as clarified in the following lines.

In vertebrate blastulae developing from mesolecithal holoblastic eggs, the vegetal blastomeres loaded with yolk are still capable of moving inwards and are involved in the formation of the archenteron wall (Fig. 2C). As a result, the embryos close up easily, more or less rapidly, helped by the epiboly of the epiblast. In these embryos a lamina of vitelline hypoblast does not organise. In some phyletic lines, during evolution, the amount of yolk in the egg tended to increase more and more and the eggs tended to become telolecithal and meroblastic as a result of that increase. In these cases the yolk accumulated from the vegetal pole to the blastoporal area. Consequently, the division of the blastomeres in that region required much more time, and it was increasingly difficult for these cells to move inwards and form the archenteron wall. This brought about a delayed closure of the embryo ventral side. Pilato (1994, 2003) hypothesised that the negative effect produced by the delayed closure did not take place where a new adaptation nullified it (and offered new advantages, such as the appearance of extraembryonic membranes). The new adaptation consisted of the early organization of the vitelline hypoblast lamina from a part of an ectomesenchyme directly deriving from the primordial ectomesenchyme of a very remote invertebrate ancestor (Fig. 4B-C). The vitelline hypoblast lamina closes the embryo temporarily and promptly and, thanks to the never lost original ability of the primordial ectomesenchymal cells to digest, it enabled an easy utilization of the yolk which no longer can be carried inside.

According to the "theory of the endoderm as secondary layer" (Pilato, 1992, 1994, 2003, 2007), the primordial ectomesenchyme is considered as the material which first moved inside producing the transformation of a monoblastic ancestor (Blastaea) in the first diploblastic organism. It moved inside to offer support to the delicate body wall but inherited the production of germ cells (which in this way are more protected) and the primordial ability to digest. Later, during phylogenesis, it gave rise to the extant mesoderm and, from a part, to the embryonic endoderm (Fig. 4B,C).
This hypothesis had a recent confirmation. In fact the existence of a primordial bipotential germ layer, or at least cell population, called "mesendoderm" (Rodaway & Patient, 2001), sometimes also called "endomesoderm", has been hypothesized by other authors not only on the basis of morphological data, but also on the basis of gene expression (Rodaway & Patient, 2001; Croce & McClay 2010). To this ancient germ layer, and to its derivatives, a crucial role in patterning the early embryo has been attributed (Croce & McClay, 2010; Yamanaka *et al*., 2010).

It is evident that the so-called "mesendoderm" can be considered equivalent to the "primordial ectomesenchyme" presented in the "theory of the endoderm as secondary layer" (Pilato, 1992) which also provides a hypothesis about the phylogenetic origin of that cellular mass.

As mentioned above, from the primordial ectomesenchyme PGCs and the extant mesoderm derived, and the digestive endoderm specialised and canalised. If so, a mesodermal origin should be attributed to PGCs.

This hypothesis provides a new interpretation of several previously unexplained facts of vertebrate embryogenesis and makes their phylogenetic significance clear. It allows us to understand why PGCs are recognizable earlier than the settlement of the digestive endoderm starts; this is simply because phylogenetically the specialization of germ cells preceded the specialization of the digestive endoderm. Actually germ cells were already produced by the very remote monoblastic ancestor of the metazoans, and also by the flagellate colony from which that ancestor derived, i.e. in organisms in which certainly a digestive endoderm was not specialised.

In the vertebrates that organised the vitelline hypoblast lamina, thanks to the formation of this structure, a part of the presumptive endoderm (which was due to form from the yolk-laden portion of the egg) no longer needed to cellularise and shift inwards to form the archenteron wall, with no harm to the embryo. This portion of the presumptive endoderm, no longer involved in the formation of the intestinal epithelium, specialised to form the extant extraembryonic membrane called the perivitelline syncytium (Fig. 4B-C). Only a small part of the presumptive endoblast, placed near the blastopore, is poor in yolk and, as mentioned above, forms the "embryonic hypoblast" (Fig.4B,C) which still moves inwards and preserves the function of developing the intestinal epithelium.

It is reasonably presumable that a record of these ancestral processes canalized during phylogenesis and is present in extant

vertebrate ontogeny. A part of the vitelline hypoblast derives from posterior cells of the marginal belt of the blastodisc and induces the formation of the primitive streak through which the definitive mesoderm and the definitive endoderm settle (Eyal-Giladi & Wolk, 1970). Except for therian mammals, the perivitelline syncytium, as a reminiscence of its endodermal derivation, is slowly covered through an epibolic process with extraembryonic layers: ectoderm and mesoderm in Chondrichthyes and Osteichthyes (Fig. 9A), vitelline hypoblast and mesoderm in reptiles and birds (Fig. 9B).

In therian mammals the vitelline hypoblast enlarges inside the blastocyst cavity forming, together with extraembryonic mesoderm, the yolk sac (Fig.8A,B).

Pilato (1994, 2003, 2007) also hypothesized that the therian trophoblast has the same derivation as the perivitelline syncytium, but it cellularises in consequence of the secondary reduction of the yolk squeezed out of the blastomeres (metatherians) or of the oocyte (eutherians) (Fig. 10).

In conclusion the vitelline hypoblast lamina, in our opinion, has an ectomesenchyal not endodermal origin, and the perivitelline syncytium, and the homologous trophoblast of mammals, have an endodermal not ectodermal origin. As a consequence, the derivatives of the vitelline hypoblast lamina should be considered derived from the primitive ectomesenchyme and not from the endoderm.

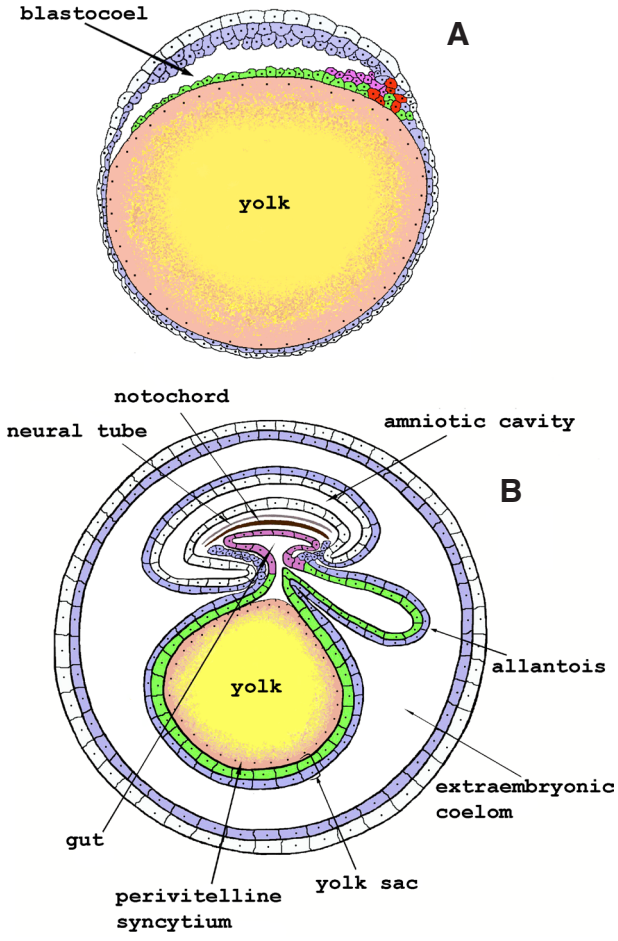

Fig. 9. Yolk sac structure. (A) *Teleostean embryo: the yolk sac is formed through epiboly of ectoderm and mesoderm only.* **(B)** *Avian embryo: the extraembryonic membranes characteristics of the amniotes are formed. The yolk sac is formed by vitelline hypoblast and mesoderm. Light-blue, ectoderm; grey, neural tube; blue, mesoderm; brown, notochord; green, vitelline hypoblast lamina; violet, digestive endoderm; red, PGCs.*

Origin of PGCs and early embryogenesis

Given the statements expressed above, in our opinion all the apparently contrasting data on the origin of PGCs in vertebrate taxa can be explained.

Origin of PGCs in vertebrates with telolecithal meroblastic eggs

Considering that PGCs appear in the vitelline hypoblast lamina and in the yolk sac wall (deriving from that lamina) and that these structures, as expressed above, have to be considered of mesodermal rather than endodermal origin, then PGCs, contrary to traditional conviction, must also be of mesodermal origin.

This is in agreement with the previously mentioned remarks of Clark & Eddy (1975). They stressed that those cells, recognised in the "yolk sac endoderm" (deriving from the vitelline hypoblast lamina), are more similar to mesodermal cells than to endodermal ones, and for this reason they hypothesised a mesodermal origin of PGCs.

The area of origin of the vitelline hypoblast considered, it becomes perfectly understandable that the nearer the precursors of PGCs are to the caudal portion of the margin of the blastodisc (or of the embryonic node), where the main inductive centre is placed, the earlier PGCs appear. In fact, the signal inducing the settlement of the vitelline hypoblast lamina starts from this centre, which, according to Eyal-Giladi (1997), is homologous with the Nieuwkoop centre of amphibians.

Pala's statement (1970) also becomes easily explainable, according to which in teleosts (*Gambusia*), at an early gastrula stage, PGCs appear in the vitelline hypoblast of the caudal half of the blastodisc. Also Gamo's (1961) statements are easily explainable, according to which in teleosts (*Oryzyas*), at an early gastrula stage, PGCs are recognizable in the unsegregated mesendoderm and occasionally also in the ectoderm.

The appearance of PGCs in the caudal portion of the primitive streak of mammals (Chiquoine, 1954; Ozdzenski, 1967) is explainable, as well as Oppenheimer's statement (1959), according to which randomly reassociated fragments of the posterior third of the "middle gastrulae" of teleosts grafted in an extraembryonic area of a host embryo formed gonad with PGCs in absence of "any endodermal" structure. The induction to form PGCs is not due to endodermal structures but to an organizer which acts early and also induces PGCs which become recognizable only later.

As a consequence of the precise relation between vitelline hypoblast, primitive streak, endomesoderm and digestive endoderm, it becomes understandable that in some cases PGCs, recognizable subsequently, appear in endomesoderm or in the digestive endoderm as noted by Hubert (1976) in reptiles.

Eddy & Hahnel (1983, page 43) reported an interesting experiment on mice and wrote: "Egg-cylinder stage embryos were separated into epiblast, extraembryonic ectoderm and primitive endoderm, and those pieces transplanted to beneath the testis capsule [..] the other pieces formed only extraembryonic tissues [..] only cells in the epiblast of 6-day mouse embryos are capable of establishing the germ line". This experiment seems to demonstrate that the vitelline hypoblast (named primary endoderm by

those authors) does not induce the formation of PGCs but, in its turn, is induced by an epiblastic centre to perform that induction.

It now seems clear that in vertebrates with telolecithal meroblastic eggs PGCs have ectomesenchymal origin and that their induction depends firstly on the Nieuwkoop centre. This centre induces the settlement of the ectomesenchyme which takes part in the formation of the vitelline hypoblast lamina which, in its turn, induces the differentiation of PGCs and the formation of the primitive streak.

If the primitive streak is considered homologous with the blastopore, according to Pilato (2003) the portion of the vitelline hypoblast lamina inducing the formation of the primitive streak has to be considered homologous with the Spemann centre of amphibians (i.e. with the area where the dorsal lip of the blastopore forms, whose inducing role is well known).

PGCs have to be considered as extremely ancient cells, and it is advantageous to shelter them. It is, therefore, logical that they arise from the primordial ectomesenchyme and it is logical that they are related to the Spemann centre which is the material that first moves inwards.

Keeping in mind these presuppositions, it is possible to explain why in most taxa with telolecithal meroblastic eggs PGCs appear in areas which during evolution have become extraembryonic.

In telolecithal eggs, the ooplasmic area, that in the ancestor producing holoblastic eggs was destined to give the Spemann centre, was pushed by the yolk, shifted near the animal pole, and "diverted" to organising early the vitelline hypoblast lamina. This novelty canalised in the descendants, and the material forming this structure retained, and still retains, its meaning and its very important primitive role. It continued, and still continues, to induce the formation of the primitive streak and to give rise to the PGCs; but since the vitelline hypoblast lamina also contributes to form the yolk sac wall, it becomes explainable that in some cases the PGCs appear in that, secondarily, extraembryonic structure. In other words, that is due to the fact that the ectomesenchyme, from which the PGCs arise, enlarges forming the vitelline hypoblast lamina which then contributes to form the yolk sac wall. That is possible the PGCs being not damaged by the longer migration necessary to reach the gonad anlagen.

During the Metazoan phylogenesis, the specialization of germ cells preceded the organization of proper gonads as also demonstrated by some primitive invertebrates where germ cells are produced but proper, well structured, gonads are lacking (Porifera, Cnidaria, some primitive Turbellaria, Annelida Polychaeta); it is therefore possible to think that in vertebrates producing telolecithal eggs, the increase in yolk amount in the egg displaced, after the induction but before the differentiation, the cells from which the PGCs would arise with respect to those from which the gonads later would develop; as a consequence, the PGCs could arise before the gonads and also far away from them (in some cases, as above mentioned, in territories secondarily extraembryonic), and a very long migration became necessary.

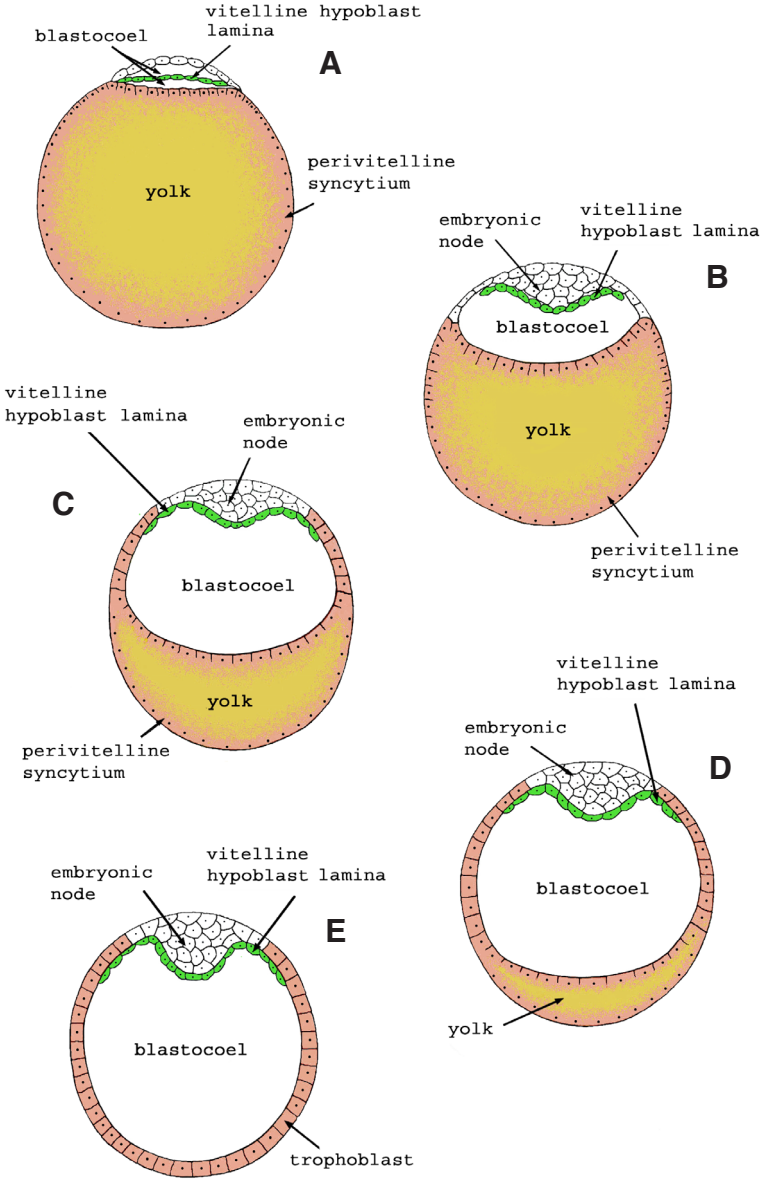

Fig. 10. Hypothetical origin of the mammalian trophoblast. *It is possible to hypothesise that the mammal blastocyst* **(E)** *derived from a reptilian blastula* **(A)** *by gradual, or sudden, reduction of the yolk. In fact the yolk is squeezed out of the first blastomeres in metatherians, and of oocytes in eutherians. The mammal trophoblast may be considered homologous with the reptilian perivitelline syncytium which, as a result of the yolk reduction, cellularises. (According to Pilato 2003, 2007).*

Origin of PGCs in amphibians

Apparently, PGCs have an endodermal origin in anurans and Caeciliidae, and a mesodermal origin (under endodermal induction) in the urodeles. In amphibians the Nieuwkoop centre forms (after egg fertilization) by shifting of maternal determinants from the initial vegetal position to an eccentric, dorsal position of the vegetal hemisphere (Eyal-Giladi, 1997). The Nieuwkoop centre induces by diffusible signals the formation of the Spemann centre, which forms the dorsal lip of the blastopore; its cells, moving inwards through the blastopore, participate in the formation of the mesoderm (Spemann & Mangold, 1924; Eyal-Giladi, 1997).

The anuran blastula is formed by more than one cell layer and, when the signal to initiate gastrulation starts from the Nieuwkoop centre, the deep blastula dorsal cells, formerly sticking to the ectoderm, get the value and the role of the

Spemann centre. They move inwards shifting along the inner surface of the more superficial cells, rapidly begin to form the mesoderm, and drag the digestive endoderm inwards.

The blastomeres destined to give rise to the PGCs are placed at the vegetal hemisphere together with those destined to form the digestive endoderm; the latter, being rich in yolk, move inwards very slowly.

Since anuran PGCs are recognizable very early, one can follow all their history. Firstly they are in the vegetal hemisphere intermingled with the endodermal cells that will give rise to the archenteron floor. From this place they migrate to the dorsal wall of the archenteron (Fig. 2 B,C), then they move along the dorsal mesentery and reach the gonad anlagen.

If, as above specified for vertebrates producing telolecithal eggs, also in amphibians PGCs do not arise from the endoderm but from a primordial ectomesenchyme, then one expects that those cells begin to migrate when the signal to move inwards starts from the Nieuwkoop centre, that signal being bound to the material derived from that phylogenetically ancient material. However, in amphibian embryos PGCs are far away from the Nieuwkoop centre (intermingled with the endodermal blastomeres), and as a consequence, the dorsal blastomeres being less rich in yolk and nearer to the Nieuwkoop centre, they are the fastest to move inwards. PGCs receive the signal from the Nieuwkoop centre later than the cells nearer to it; moreover, their migration is also slowed by the presence of the endodermal blastomeres that, conversely from that which occurs in embryos developing from telolecithal meroblastic eggs, all move inwards as well, but slowly due to the amount of yolk which they hold. As a consequence, PGCs, although recognizable very early and having ectomesenchymal origin, secondarily begin to migrate later than the mesodermal blastomeres placed nearer to the Nieuwkoop centre. Since they are not able to part quickly from the digestive endoderm, as is the rest of the mesoderm, they are still incorporated in the archenteron wall, leading to the misperception that they have an endodermal origin.

In conclusion, differently from the vertebrates with telolecithal eggs where the ooplasm has been pushed nearer to the Nieuwkoop centre, the anuran PGCs are slow in migrating. As a consequence they do not settle earlier than the digestive endoderm but behave like the extant endomesoderm that parts subsequently from the endoderm and seems to arise from it. For this reason, until now an endodermal origin, instead of ectomesenchymal, has been attributed to anuran PGCs.

Urodeles and Caeciliidae still have holoblastic eggs but, some urodeles excluded, more rich in yolk than those of the anurans; therefore those eggs are in an intermediate condition between the mesolecithal eggs of anurans and the telolecithal meroblastic eggs. All morphogenetic areas of the urodelan eggs (and therefore the blastula presumptive regions) are shifted towards the animal pole more than in anurans but less than in telolecithal meroblastic eggs (and the blastula developing from them). In consequence of this displacement, when the inductive signal to form the Spemann centre (and to initiate the gastrulation) starts from the Nieuwkoop centre, the precursors of PGCs respond to that signal more rapidly than in anurans. As a consequence, when the urodelan PGCs become recognizable they are not still intermingled with the endodermal blastomeres but they have already moved inwards and are part of the lateral plate mesoderm (Fig. 3).

Conclusions

All this considered, we think that the differences between vertebrate phyletic lines as regards the origin of PGCs are only an appearance. In our opinion in all vertebrates PGCs arose from the same cellular stem deriving from the primordial ectomesenchyme of the first diploblastic ancestor of the metazoans. It is possible to attribute the differences to variations in the distance between the area of origin of PGCs and the Nieuwkoop centre, that distance being closely connected to the yolk amount and the yolk distribution in the ooplasm. The seeming differences are also a consequence of the more or less early appearance of the characters that allow us to recognize PGCs.

We wish to stress that recently Pilato (2000) presented facts that lead us to think that also in Placozoans, Porifera and Cnidaria the germ cells arise from cells deriving from a primitive ectomesenchyme. Since PGCs in vertebrates seem to have the same origin, and since it is evident that in the other phyla the germ cells have mesodermal origin, one can conclude that, in contrast to traditional thought, the origin of the germ cells appears to be unique in all pluricellular animals. Extavour & Akam (2003) agree with this statement, which aligns fully with the entire animal phylogeny based on the “theory of the endoderm as secondary layer”.

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