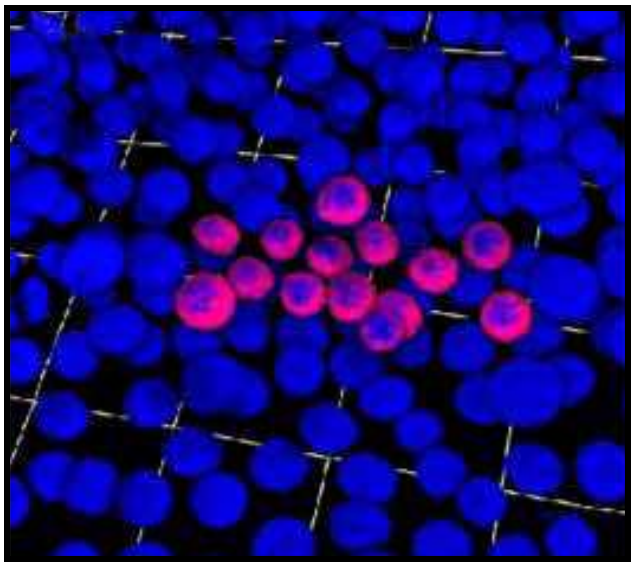
Tätigkeitsbericht für die „Austrian Marshall Plan Foundation“

Germ cell specification

(Epigenesis, Preformation)

in

Drosophila melanogaster*, *Mus musculus* and *Parhyale hawaiensis



eingereicht von Elisabeth Maritschnegg

am 3. Dez. 2008, Graz

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Cover Picture: Extavour, C.G. (2005). The fate of isolated blastomeres with respect to germ cell formation in the amphipod crustacean *Parhyale hawaiensis*. *Dev. Biol*. **277**:387-402

Abstract

Germ cell specification is the process where cells in embryogenesis develop into a distinct direction, either into somatic, or into germ cells. This is a very important topic in Developmental biology, as it gives information about evolution. There exist two distinct modes of germ cell specification, epigenesis and preformation. I want to explain these two modes on the example of *Drosophila melanogaster*, which specifies its germ cells by preformation, and the epigenesis mode in *Mus musculus*. There exist many differences, but one conserved aspect is transcriptional repression, respectively the repression of the somatic program. I included *Parhyale hawainesis* in this thesis, because I did research on this organism in the Extavour lab, department of organismic and evolutionary biology, Harvard University, Boston in summer 2008. Interestingly *Parhyale* is the first organism, which normally specifies its germ cells exclusively by preformation, but can switch to an epigenetic program. This was proofed when germ cell precursor ablated animals without PGCs developed completely normal gonads again.

1. Introduction

The adult human body is made up of billions of cells. Most of these cells are working to keep a single individual alive and healthy, but a small percentage of cells are working for many future generations – the germ cells. [1]

All sexually reproducing organisms arise from gametes, which are specialised haploid cells, in animals either sperm or an egg. They are produced by meiosis of germ cells and are the only cells that can generate an entire new organism. [2] Meiosis is a process of reductional division and in comparison to mitosis, the number of chromosomes is being reduced from diploid to haploid, when DNA replication, followed by two rounds of division has occurred. It results in the formation of gametes and during fertilization a new diploid cell is created. [3]

In most organisms, gamete production occurs lifelong, by asymmetric division of germ stem cells, so that one product remains a stem cells, while the other one differentiates [4] and begins gametogenesis. [5]

Lewis Wolpert, in his classic text “The Triumph of the Embryo” remarked that gastrulation, not birth, death, or taxes, is the most important event in our lives. [6]

From 30 trillion cells in a human body, just a very small percentage specifies to germ cells, but it’s those cells, which play a unique role in gamete production, heredity and evolution. An average American has 2 to 3 children, which means that 2 or 3 cells will contribute to the next generation and have the potential to develop into a whole new organism [7]

Therefore germ cell specification, the process where germ cells are set aside from somatic cells, is one of the main topics in developmental and evolutionary biology. Still, the processes involved are not completely understood.

2. Germ cells

More than 100 years ago, Weismann was the first one to recognize the role of germ cells in the continuity of a species. [8] At fertilization, when egg and sperm fuse, most of the cells will give rise to the soma and those cells eventually die, but the primordial germ cells will provide the everlasting germline cycle and will go on and on for generation to generation. [9, 10]

I want to give a few definitions:

Germ cell: Any precursor cell that can give rise to gametes. [11]

Germ line: genetic material transmitted from one generation to the next through the gametes. [11]

Primordial germ cells (PGCs): diploid germ cell precursors [12]; in many organisms motile and migrate to the somatic gonad [7], where they become irreversibly committed as germ cells. [12] They are highly specialized and unable to give rise to any other cell type than germ cells when transplanted from one embryo to the next in mice or flies. [13, 14]

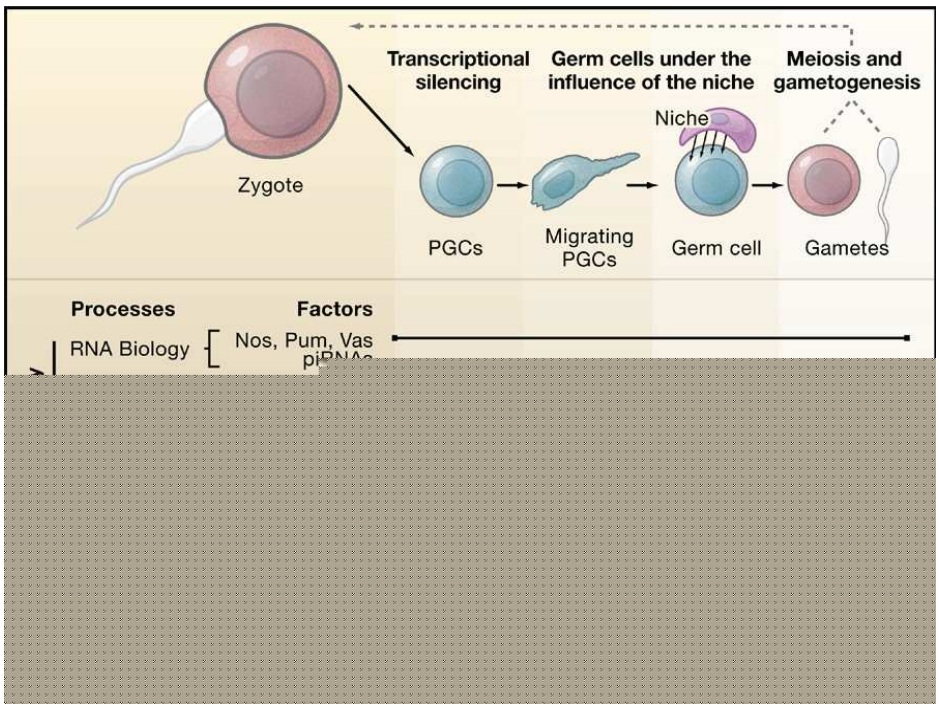

Fig.1: The life cycle of a germ cell and conserved processes, which affect germ cell formation and maintenance and the expression of factors needed at specific stages in the fly, worm and mouse. [9]

The general steps which a germ cell goes through are already mentioned in the text above. I just want to add that niche is understood as specific somatic cells, which a subset of PGCs becomes associated with and protects the germ line stem cells from differentiation. [15]

Its signalling is required for the differentiation of germ cells into specialized gametes.

RNA biology includes the regulation of RNA translation, stability and processing. One conserved aspect among many species, needed for germ cell specification, is transcriptional silencing, respectively to prevent them from somatic fate, they repress the transcriptional

programs for somatic differentiation. [9] Later on in development of germ cells, there emerges a chromatin-based mechanism of transcriptional repression [16], but a definitive mode has yet to be identified in flies. [17] The processes and factors shown in picture1 will be mentioned later on.

2.1 How can germ cells be identified?

One can distinguish somatic and germ cells on histological or molecular aspects. Their histological characteristics are a large round nucleus, single large nucleolous, cytoplasm relatively clear of organelles,

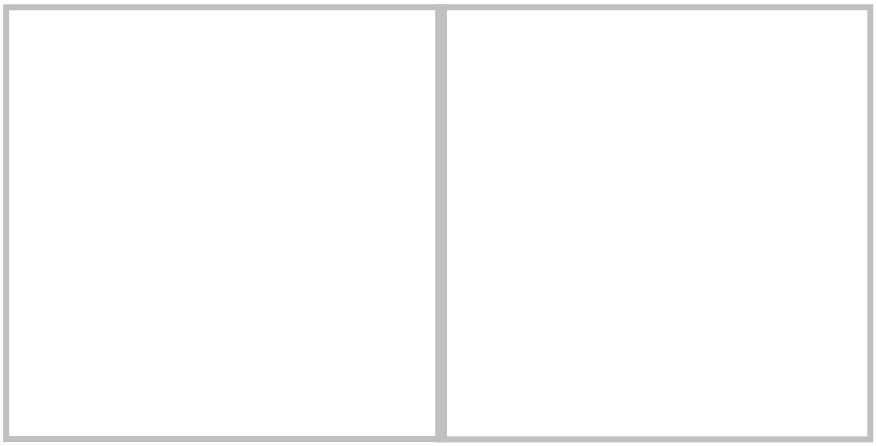

Fig.2: PGCs detected with vasa protein in Parhyale hawainesis. (2A) High magnification of PGCs, which shows that Vasa protein is perinuclear. (2B) A 3D reconstruction of the embryo in dorsal view, showing the germ disc with approximately 300 cells and 15 PGCs clustered together. [19]

and granular cytoplasmic material. This material is often called „nuage“ and is a way to identify PGCs early in development.

Molecular markers used for identification are alkaline phosphatase, or the products of germ cell-specific genes, like of the vasa gene family (Fig.2). [7]

Another difference is, that germ cells remain transcriptionally inactive 3-6 stages longer (differs from species to species). [18]

In this sense, germ cells can be seen as the stem cells of the species and are the means by which species form and change in evolution. In humans they are also the vehicles for inherited diseases. Despite their philosophical and medical importance, there’s still little known about the mechanisms whereby they arise during development, or how they differentiate into the gametes. [2]

3. Germ cell specification

Germ cell specification addresses how the fundamental distinctions between germ cells and somatic cells are initiated and maintained throughout development and is therefore a very important topic in developmental biology, to do research on. [20] They are set aside in development early, so they don’t undergo differentiation and specialization stages, where they would obtain somatic fate and confine their potential to become a whole new organism. [21] There exist fundamental differences in early development of animals, because of that it’s necessary that the robust mechanisms involved in the specification of the germ line aren’t

conserved, although it's one of the most crucial events. [16, 22] One conserved aspect is the repression of programs of somatic differentiation, [5] which will be explained in further chapters.

The establishment of the germ cell lineage in animals falls under one of two apparently distinct mechanisms, Epigenesis and Preformation.

3.1 Epigenesis:

After a few divisions of a fertilized mammalian egg, the produced cells are pluripotent, so they have the potential to differentiate into any cell type in the body, also in germ cells. [23, 24] These undifferentiated cells receive inductive signals from neighboring tissues, controlled by zygotic genes. They respond by differentiating as germ cells, but can just be observed relatively late in development. [7] This mode has only been examined in vertebrates, such as in mice and axolots, [25, 26, 27, 28, 29, 30] but appears to be the most common and possibly ancestral mechanism of metazoan germ cell specification. [7]

3.2 Preformation:

In the preformistic way, germ cells can be identified very early in embryogenesis. [7] A specialized maternal cytoplasm, the germ plasm, forms in the egg. It is inherited by only one or a few cells, which become PGCs and enter the germ lineage. It contains specific maternally provided germ cell determinants, which can be produced and localized either autonomously by oocytes, or by associated nutritive cells and is asymmetrically partitioned during oogenesis and/or after fertilization. Germ cell specification via Preformation has been observed in most model organisms, like Drosophila melanogaster, C. elegans and the zebrafish Danio rerio. [7, 19, 5, 31] The germ line determinants contained in germ plasm seem to be highly conserved, [32] but the mechanisms involved are quite different. [19]

4. Drosophila melanogaster - Preformation

An example for germ cell specification via preformation is the model organism Drosophila melanogaster.

After the egg is fertilized, a series of rapid, synchronized nuclear divisions take place and form a syncytium, the early Drosophila embryo. [33] Its the germ cells which become formed first, when they migrate to the posterior pole of the embryo, where they reach the germ plasm. [34] The next step is cellularization, where the nuclei become surrounded by cell membranes. [35] In contrast to somatic nuclei, these cells stop synchronous divisions and are therefore committed to germ cell fate, as shown by transplantation experiments. [14] These four or five primordial germ cells are called "pole cells" and their cellularization and

formation depends on the right timing to reach the posterior pole as well as on the germ plasm itself. [36]

4.1 Germ Plasm:

During oogenesis RNAs and proteins are synthesised by nurse cells and transported to the oocyte through cytoplasmic bridges. [7] There, more exactly at the posterior pole, the germ plasm, a spezialised, maternally provided cytoplasm, is sequestered before fertilization. [38] Information about the composition of the germ plasm comes mainly from the study of maternal effect genes [8] and electron microscopic analysis, which have shown, that it contains electron dense structures, called polar granules. [37] They contain germline specific proteins like Vasa, Oskar and Tudor and the mitochondrial 16S large rRNA, although the exact composition of the granules and the germ plasm is yet unknown. [38, 39, 40] The polar granules can be detected with vasa protein and are continuously present in the germ line. After fertilization they arrange as large clusters, often associated with mitochondria. When the pole cells form, two electron-dense structures called nuage and nuclear bodies, appear. Nuage is related to the germ cell nuclei throughout the life cycle, where else nuclear bodies disappear, but later recur in the ovarian nurse cell nuclei. [41]

The germ plasm habours germ cell determinants what leads in germ cell fate of every cell which contains that cytoplasm. There exist two experiments that prove the presence of determinats. First, the transplantation of the germ plasm to another site, but the pole plasm, leads in PGCs at that site. [42] Second, so far twelve genes have been identified, to be necessary for germ plasm formation: oskar, vasa, valois, tudor, cappuccino, spire, staufen, pipsqueak, mago nashi, orb, homeless, and tropomyosin II. If females lack any of these genes, they produce embryos without polar granules, which can’t form PGCs. This class of mutations is often named as “grandchild-less” as the descendants of the females are sterile. [8] Functions which have been ascribed to the germ plasm and its components are:

- Localization and translation of maternal RNAs and proteins and their protection from degradation [43, 44]
- A type of cellularization which is specific to PGCs [45]
- Global transcriptional silencing [46]
- Germ cell migration [47]

4.2 Transcriptional silencing:

To prevent the germ cells to undergo a somatic fate, transcriptional silencing is very important. Somatic cell nuclei initiate mRNA transcription about 1 hr after fertilization, where else germ cell nuclei become transcriptionally repressed as soon as they from and start accumulating zygotic mRNAs about 3 hours post fertilization. [46]

There exist two independent modes of transcriptional silencing: the earlier one bases on the direct inhibition of RNA polymerase II and the second and later one is chromatin-based and includes nucleosome modification. [48]

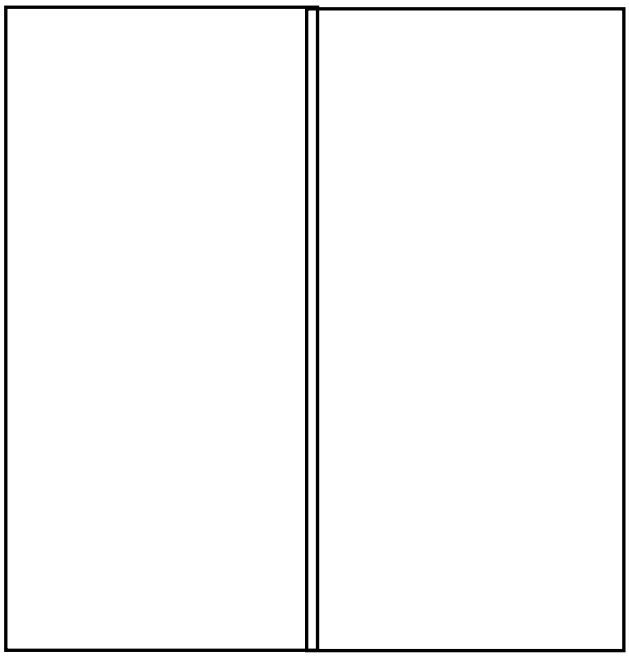

Fig.3: Mechanism of Germline Formation in Drosophila
Determinants are preformed during oogenesis, germ plasm (red) is partitioned asymmetrically in the oocyte. Inhibiting of transcription and chromatin modifying are required. [5]

During transcription, the phosphorylation of a repeat motif of the carboxy-terminal domain (CTD) on Serine at position 5 is essential for transcription initiation and on Serine 2 for elongation. However in germ cells of Drosophila P-Ser5 is reduced and P-Ser2 is completely absent, what leads to the conclusion that transcription is blocked between those two steps. [46]

The chromatin-based mode can be explained by reduced levels of H3-K4me2 (dimethylation of lysine 4 on histone H3). This is one of the methylation modifications, characteristic for transcriptionally active chromatin. [17, 48]

Three localized RNA's involved in the early germ cell specification, especially in transcriptional silencing are germ cell-less (gcl), nanos (nos) and polar granule component (pgc). [49]

Gcl is localized peri-nuclearly and seems to be a determinant of germ cell formation in the germ plasm. It is important for early specification, where it plays a role in transcription silencing. This might work by association with chromatin binding proteins, which translocate to them periphery of the nucleus, furthermore they can interfere directly with Polymerase II.

Germ cells mutant for gcl: [49, 50, 51, 46]

- have a reduced germ cell number,
- causes premature transcriptional activation in pole cells
- they accumulate zygotic transcripts and P-Ser2

Nanos is a germ plasm component and cytoplasmic protein, which acts as translational repressor. Indirectly through this function it represses transcription by inhibiting components of the transcriptional machinery itself. In both cases it works together with its partner pumilio (pum).

Germ cells mutant for nanos (and pum): [52, 53, 54, 55]

- Divide too early
- clump together
- genes normally restricted to the soma, become expressed
- don't migrate correct
- germ cell specific genes become transcribed too early

Pgc is maternally provided noncoding RNA. It has also got a direct effect on the RNA polymerase, as it inhibits a kinase that phosphorylates the CTD, what constricts the transition from initiation to elongation complex.

Germ cells mutant for pgc: [56, 55, 57]

- Don't migrate correct
- clump together
- many die prematurely
- genes normally restricted to the soma become expressed
- RNA polymerase activity and histone methylation are similar to somatic cells – so is the transcriptional activity

Gcl seems to be required earlier than nos/pum and pgc as it also affects germ cell formation. The primary function of nos/pum is to maintain germ cell identity. The primary role of pgc is transcriptional silencing and is therefore necessary a little later than the others. They all work in different pathways and lots of other genes are involved in germ cell development in Drosophila (for a list of those genes see Review [58]) so that the unique mechanisms, which inhibit somatic differentiation, can occur. [58]

Germ cell specification is followed by the migration of the PGCs to the somatic gonade, where at the end of embryogenesis the embryonic gonad forms. [8]

5. Mus musculus – Epigenesis

The mouse specifies its germ cells via epigenesis, so there are no preformed germ cell determinants involved. The PGCs arise during gastrulation, what is in comparison to Drosophila relatively late in development as it starts only after implantation of blastocysts. The primordial germ cells arise from pluripotent proximal epiblast cells of the egg cylinder at embryonic day (E)6.25. [59] These cells have got the potential to develop into germ cells, as well as into somatic cells like the majority of them does. [24] Therefore this mode of germ cell specification is often referred to as the stem cell model.

The pluripotent cells are surrounded by the extraembryonic extoderm (ExE) and visceral endoderm (VE). They are not crucial for the germ cell specification itself, but for the acquisition of competence. [60] These are the sources of signals like Bone morphogenetic protein 4 (BMP4), BMP8b and BMP2. [25] With loss of these molecules, the cells lose the ability to develop into PGCs. [61] The transcriptionally active pluripotent cells can respond to those signals and acquire competence to develop into germ cells, [60] which results in the expression and repression of different genes.

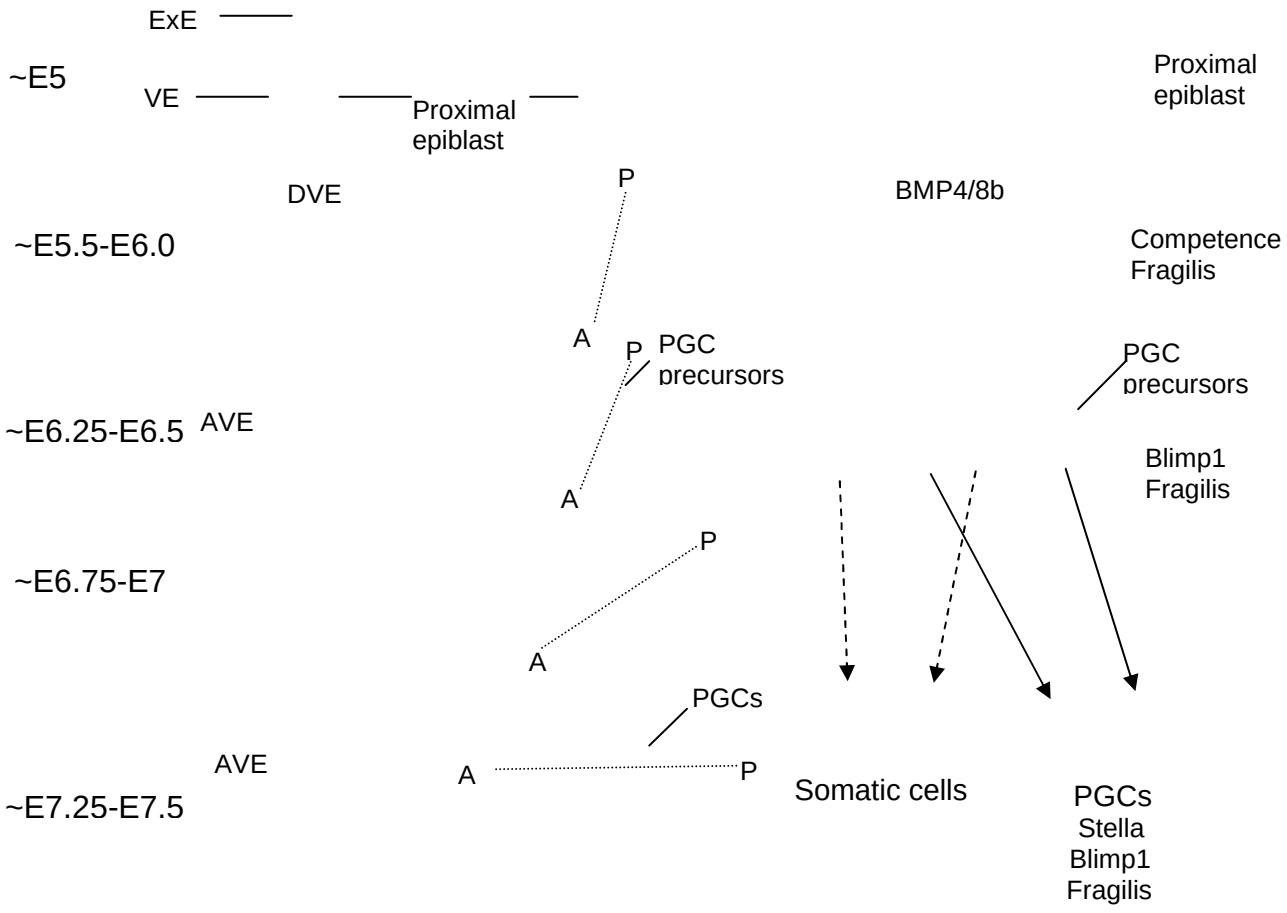

Fig.4: Early development of Mus musculus from E5.0 till E7.5 [60]

These signals seem to last at least until E 6.5 and so the number of Blimp1 positive cells, which is a marker for PGC precursor cells and will be explained further later on, increases from 6 to 16 between E6.25 and E6.5. Around E7.25 a cluster of about 40 PGCs forms in the extraembryonic mesoderm, in a structure called allantois. [62]

One of the genes involved in the transcriptional program of the PGCs is fragilis . It encodes for a member of a transmembrane protein family. When the cells respond to the signals mentioned above, they start to express fragilis, and are then able to develop into PGCs. As BMP4 homozygous mutants lack expression of fragilis, and it is reduced in heterozygous ones, it seems to depend on the amount of BMP4. [24] At the same time BMP triggers serine phosphorylatoin of Smad1/5/8, which are transducers of BMP4 signaling, and are expressed in the epiblast. [63] At gastrulation the expression of fragilis shifts to a region where the PGCs can be detected afterwards. [24]

5.1 Transcriptional repressor Blimp1

At E6.25 six of the fragilis positive cells start to express Blimp1. Its cells which are on the prospective posterior proximal site and which are in direct contact to ExE. [62] This fact indicates, that their might be a connection between the number and location of PGCs and anterior-posterior axis formation. [60] It's a key transcriptional regulator of PGC development and cells of that region, expressing Blimp1 are committed to germ cell fate.

On the one hand it's responsible for the repression of somatic program, what is crucial for germ cell specification in many model organisms, like Mus musculus. On the other hand it makes it possible for germ cells to establish their specific characteristics. [62] Its important role is evident as its involved in the repression of nearly all the genes down-regulated in PGCs in comparison to somatic cells. However it's not involved in the up-regulation of half of the genes. [64] Blimp1 is a protein with a Pr/Set domain, five zinc fingers, a proline-rich region and a C-terminal acidic domain. [65]

Cells mutant for Blimp1 show deficiencies in PGC specification very early and establish only about 20 PGC-like cells in which the repression of Hox genes and the activation of PGC specific genes, like stella are restricted. [60]

Recent studies found Blimp1 associated with the arginine-specific histone methyltransferase Prmt5. This complex may play a role in maintaining the germ cell lineage and during migration, but it's still unknown, if Prmt5 itself, or through the association with Blimp1, has a part in the specification. [60]

Quantitative single-cell gene expression profiling of several genes expressed in Blimp1 positive cells between E6.75 and E.8.25 show a developmental heterogeneity in those cells. Especially at earlier stages a certain part of them shows Hoxb1, but not Sox2 expression.

Sox2 is a key gene for pluripotency and since at later stages the expression is contrary, it seems that the Blimp1 positive cells first have properties more similar to somatic cells. Then they turn of their somatic program and reacquire the potential of pluripotency. [66, 67]

Stella positive-cells first appear around E7.25. It's a small nuclear cytoplasmic shuttling protein with a SAP-like domain and seems to be germ cell specific as it is still expressed in PGCs when they migrate. [24] The stella positive cells show repression of Hox genes.
The repression of the region-specific homeobox (Hox) genes is critical for the germ cell specification, since they cause differentiation of cells into somatic lineage and are therefore highly expressed in somatic cells. [57] This includes Hoxb1, Hoxa, Lim11 and Evx1. [68]

6. Parhyale hawaiensis

Parhyale hawaiensis is an amphipod crustacean of the class Malacostraca and a relatively new model organism to do comparative studies and genetic analysis on, because it's ease of laboratory culture, molecular manipulation and has a well-defined cell lineage during development. [69]
The two following cleavages after fertilization are slightly unequal what makes one of the blastomeres at four-cell stage a little bit smaller than the others. The third cleavage is very unequal and the products are four macromeres and four micromeres. [69] The nomenclature of them can be found on the figure 5.

This third cleavage exclusively specifies the three germ layers and the germ line. The smallest micromere is called "g" and can be identified relatively easy, as the smaller of the two micromeres don't touching each other in the middle. This g micromere is the source of the future germ cells, although it's yet unknown, how it acquires germ cell fate, as it also lacks nurse cells. Different experiments lead to the conclusion that a germ line determinant is localized in the smaller one of the cells at two-cell stage and is segregated only in the precursor cell of the g-micromere until the fourth cleavage. [19]

Fig.5: Parhyale hawaiensis embryo at 8-cell stage [69]

In the study I refer to, E.G. Extavour used Vasa protein as a marker to identify primordial germ cells in P. hawaiensis from their specification at the eight-cell stage until the end of embryogenesis. The cells identified by the cross reacting vasa antibody correspond to those

cells identified in previous cell lineage studies as being the exclusive descendants of the “g” micromere. Extavour was able to show, that only one cell at the two-cell stage, the cell that will give rise to the germ line founder cell at the eight-cell stage, contains a cytoplasmic germ line determinant. She also demonstrated that acquisition of germ cell fate is autonomous and unique to this germ cell precursor, and that other blastomeres cannot give rise to germ cells even if the germ cell precursor is ablated. [19]

Not much is known about the germ cell specification in *Parhyale h.* itself, but *nanos* and *vasa* mRNA seem to be involved. Recently another mRNA was found to be important even before *nanos* and *vasa, beta-catenin.* It can already be found in the one-cell stage and is again segregated in the g-micromere, respectively in its precursors. *Vasa* mutant germ cells stop dividing after gastrulation and in cells mutant for *nanos* the migration is reduced also. [71]

Fig.6.: Adult sexually mature male and female *Parhyale hawaiensis [70]*

Parhyale hawaiensis is not as well understood as model organisms like *Drosophila melanogaster*, but many aspects support specification of PGCs by preformation.

- The germ plasm is a sign for preformation and is present and can be identified in the Parhyale embryo at the one-cell stage clearly.
- One can observe the segregation from specialized cytoplasmic components to the g micromere in living embryos and through the localization of β-catenin mRNA. During the one-cell stage it corresponds to the morphologically distinct cytoplasm and throughout the following divisions it is segregated asymmetrically to the cell that will later correspond to the g-cell. [19]
- After fertilization an aggregate of mitochondria, also called mitochondrial cloud, forms and segregates to the g-micromere during the first three cleavages. This cloud is often associated with the germ plasm, and is therefore just observed in animals using preformation. [72, 73, 74]

In experiments of C. Extavour, they ablated the g-micromere, the only source of PGCs, and by that expected to become embryos without PGCs. As control for the completely absence of the g cell they used the germline marker *vasa* and control based on morphological aspects. They proofed that the animals lacked PGCs, but still more than 90% of the males and females were fertile when raised to adulthood. This means, that

Parhyale is able to generate gonads in the absence of PGCs. This is the first time one has observed that an animal which normally specifies its germ cells exclusively by preformation, can generate functional gametes through a post-embryonically epigenetic mode. [75]

Evolutionary studies assume that epigenesis is ancestral within the metazoans, as well as in the lineage leading to the arthropods and preformation is a derived trait. [7] One theory of the evolution of preformation is a heterochronic shift in expression of genes required for gametogenesis, like *vasa* and *nanos*, to earlier stages. [76, 77] In *Parhyale* it seems that with the upcoming preformation, the epigenetic mode hasn't been lost. In contrast to for example *Drosophila*, where this is not the case, but there might still be other animals that can use preformation and Epigenesis.

At the moment the source of the replaced germ cells is not known but it could be that *Parhyale* is able to reverse or manipulate the crucial steps that distinguish somatic cells and germ cells. [75]

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