Summary of professional accomplishments
(Autoreferat)

1. Name and Surname:

Anna Piliszek

2. Diplomas, scientific degrees -

2006 - PhD in agricultural sciences in the field of animal science. Institute of Genetics and Animal Breeding of the Polish Academy of Sciences in Jastrzębiec. Title of the dissertation "Possibilities of chimerism arising between cleaving mouse embryos and fetal cells"; Supervisor: Prof. J. A. Modlinski.

2001 - MSc in Biology in the field of general biology. Faculty of Biology, University of Warsaw. Title of the dissertation "The role of histone acetylation in the regulation of transcription in the first cell cycle in murine oocytes". Supervisor: Prof. AK Tarkowski.

3. Information on previous employment in scientific / artistic units.

01.03.2006 - 31.08.2006 biologist, Department of Experimental Embryology, Institute of Genetics and Animal Breeding of the Polish Academy of Sciences

From September 1, 2006, until now, assistant profesor (adiunkt), Department of Experimental Embryology, Institute of Genetics and Animal Breeding of the Polish Academy of Sciences (including scientific leave in 2006-2010).

2006-2010 Postdoctoral research fellow, Sloan Kettering Institute, New York, USA

4. Indication of achievement [1] resulting from art. 16 sec. 2 of the Act of 14 March 2003 on academic degrees and academic title, and on degrees and title in the field of art (Journal of Laws of 2017, item 1789)

a) title of scientific achievement:

Mechanisms of epiblast and primitive endoderm differentiation in mouse and rabbit embryos

b) (author / authors, title / titles of the publication, year of publication, name of the publishing house, publishing reviewers)

1. Płusa, B. *, **Piliszek, A. *,** Frankenberg, S. *, Artus, J., Hadjantonakis, AK., Distinct sequential cell behaviours direct primitive endoderm formation in the mouse blastocyst (2008)

* first author / equal contribution of co-authors

MNiSzW point = 40, IF = 6.812, citations = 269

2. **Piliszek, A. #** , Kwon, G.S. and Hadjantonakis, A.-K. (2011) Ex utero culture and live imaging of mouse embryos. In: Vertebrate Embryogenesis. Methods in Molecular Biology: Embryological, Cellular and Genetic Methods.

corresponding author

Methods in Molecular Biology. 770: 243-57, Ed. FJ Pelegri. Ed. Springer (book chapter, no IF available), citations = 13

3. Artus, J. *, **Piliszek, A. *,** Hadjantonakis, A.-K. (2011) The primitive endoderm lineage of the mouse blastocyst: the sequential transcription factor activation and the regulation of differentiation by Sox17. Developmental Biology 350 (2): 393-404.

* first author / equal contribution of co-authors

MNiSzW point = 35, IF = 4.069, citations = 106

4. **Piliszek, A.** #, Madeja, Z., Plus, B. # (2017) Suppression of Erk signalling abolishes primitive endoderm formation but does not promote pluripotency in rabbit embryo. Development 144 (20): 3719-3730.

corresponding author

MNiSzW point = 40, IF = 5.413, citations = 10

5. **Piliszek, A.** #, Madeja, ZE (2018) Pre-implantation development of domestic animals. In: Cell Fate in Mammalian Development. Current Topics in Developmental Biology. Vol. 128, pp. 267-294; Ed. Hadjantonakis, A.-K., Plusa, B.

corresponding author

MNiSzW point = 40, IF = 3.11 (for 2017, the current one is not available), citations: 0

c) discussion of the scientific / artistic purpose of the above work / work and results achieved, discussing their possible use.

Explanation: in the Polish text, in addition to the Polish nomenclature, English terminology also appears in brackets. This is due to the fact that the majority of contemporary literature on the subject is published in English. While striving to use correct language in my native language, I also wanted to make it easier for the reader, no doubt better acquainted with the English nomenclature. Therefore, also the abbreviations used also come from English, because they are widely used in the scientific world.

INTRODUCTION

Early stages of development of placental mammals are the time of **differentiation of the first cell lineages** - pluripotent **epiblast** (EPI), and extra-embryonic **Pr**imitive **E**ndoderm (PrE) and **trophectoderm** (TE). Proper differentiation and segregation of these lineages is a prerequisite for further development, and errors in this process may result in inhibition of development and death of the embryo at the stage of implantation. Epiblast cells give rise to all fetal tissues as well as to embryonic stem cells in in vitro culture. The extra-embryonic lineages – trophectoderm- responsible for the implantation, and the primitive entoderm - co-constituting the fetal membranes - do not participate in the formation of the body of the future organism, however, because the development of placentae takes place inside the mother's body, their correct differentiation is necessary to sustain development. The primitive endoderm is also the source of signals that direct epiblast differentiation in the early postimplantation stages.

Individual mammalian development begins at the moment of fertilization, when the first cell of the organism arises - zygote. This cell is fully totipotent, i.e. capable of creating all cell lineages and tissues. In the course of further development, the zygote divides giving rise to all cells of the body, which in time lose the totipotency and undergo differentiation, gaining properties specific to particular lineages and tissues.

Initially, it was thought that the differentiation of the primitive endoderm and epiblast is directly dependent on the location of the cells in the inner cell mass (ICM). Indeed, at the stage of implantation of the mouse embryo (4.0-4.5 days post coitum, dpc), PrE cells are arranged in one layer adjacent to the blastocyst cavity, while EPI cells form an aggregate surrounded by PrE on one side and TE on the other side (figure 1). What's more, at this stage (4.5 dpc), the transfer of cells from these two layers into another embryo region allowed to prove that they are already differentiated (Gardner and Rossant, 1979). An additional functional confirmation of this theory was the fact that the embryoid bodies resulting from the aggregation of embryonic stem cells (ESCs) spontaneously formed a structure in which the outer layer differentiated towards the primitive endodermal derivatives (Becker et al., 1992, Martin and Evans, 1975, Murray and Edgar, 2001).

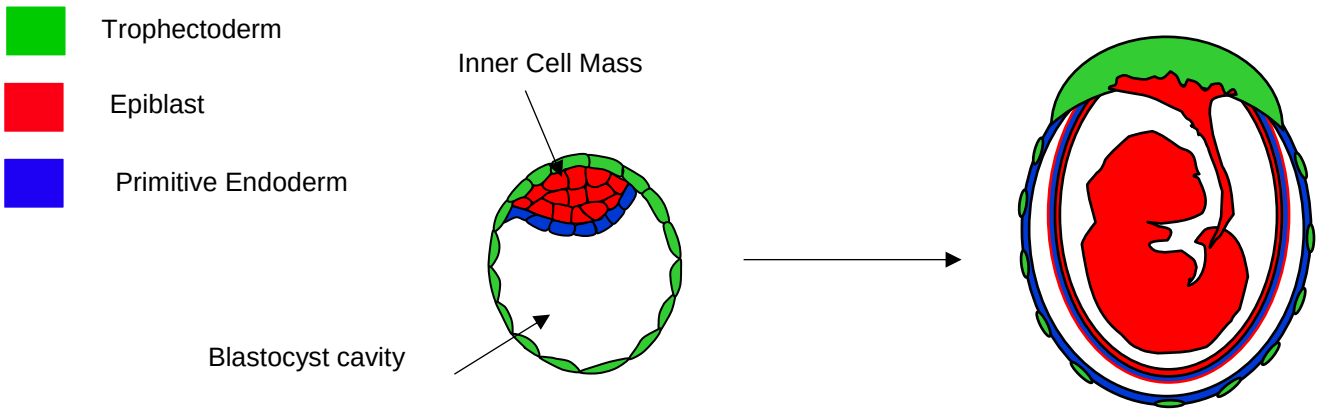


Fig. 1 Schematic representation of the murine blastocyst about 4.0 day of development, along with the presentation of the relationship between early embryonic lineages and subsequent fetal tissues

Research conducted by the group dr Janet Rossant also showed that the precursor cells of the EPI and PrE lineages are located in the ICM of mice already at the stage of the average blastocyst, about 3.5 days of development. It was found that they are not arranged in two distinct layers, but form a seemingly random mosaic in which the expression of the transcription factors Nanog (EPI) and Gata6 (PrE) are mutually exclusive - this arrangement is called "salt-and-pepper" (Chazaud et al., 2006, Rossant et al., 2003). (figure 2). The fact that cell were already differentiated at this stage was confirmed by labeling single cells of the ICM, and then analyzing their subsequent fate. Because each cell was involved in the creation of the EPI or PrE line, and never on both lineages, it was found that these cells, already at the mid-blastocyst stage (3.5 dpc) are differentiated, and only later segregate into two layers (Chazaud et al. 2006).

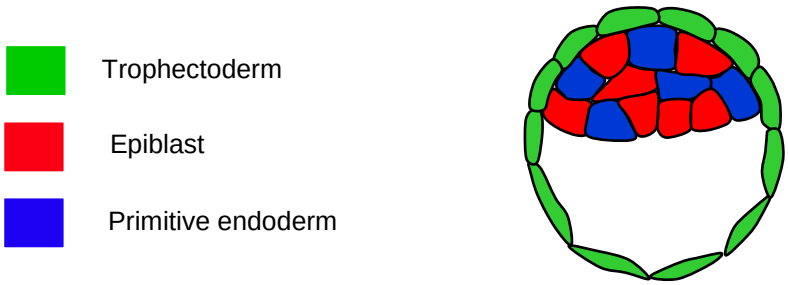


Fig. 2 Schematic representation of the murine blastocyst about 3.5 day of development, at salt-and-pepper stage

The works included in my scientific achievement present a detailed analysis of **selected mechanisms for the differentiation of the epiblast and primitive endoderm lineages in mammalian-mouse and rabbit - embryos.** I became interested in this topic during a postdoctoral training in the laboratory of Dr. Anna-Katerina Hadjantonakis at the Sloan Kettering Institute in New York. The presented works are in part the result of research conducted during this period. In recent years, I continued research on this subject, extending it to include comparative research using another model mammal species, i.e. the rabbit.

1. Płusa, B. *, **Piliszek, A.** *, Frankenberg, S. *, Artus, J., Hadjantonakis, AK. (2008) Distinct sequential cell behaviours direct primitive endoderm formation in the mouse blastocyst **Development** 135: 3081-3091, MNiSzW = 40, IF = 6,812, citation = 269

In order to follow the course of EPI and PrE differentiation , in the paper by Płusa et al., 2018, we examined changes in the expression of transcription factors associated with these lineages in mouse preimplantation embryos at the protein level, and we also used live imaging in *in vitro* cultured mouse embryos .

Identification of PDGFRa as a PrE lineage marker. The microarray analysis of preimplantation mouse embryos (Kurimoto et al., 2006) showed that the gene encoding the platelet derived growth factor alpha (Pdgfrα) was distinguished among the genes associated with PrE; its expression has also been found in XEN cells (extra-embryonic endoderm) (Kunath et al., 2005). The localization of the PDGFRa protein to the primitive endoderm lineage was confirmed by double immunofluorescent staining, localizong the GATA4 protein and the PDGFRa protein - both proteins were detected in the same cells in the late blastocysts mouse embryo (4.0 dpc).

In a further stage of the study, we used the existing *Pdgfra H2B-GFP* mouse line (Hamilton et al., 2003), in which the gene sequence encoding the green fluorescent protein (GFP) was combined with the human histone H2B coding sequence. This sequence was then introduced into the *Pdgfra* locus . Thanks to this, all cells in which the Pdgfra gene was expressed were visible due to expression of the GFP protein. The GFP fusion with the H2B histone protein additionally caused the localization of GFP in active chromatin / cell nuclei (due to H2B), which additionally allowed for better signal resolution and effective tracking of cell behaviour.

To confirm the utility of this line as a marker, we performed a detailed analysis of GFP reporter expression in pre- and peri-implantation embryos. We found that in the *PdgfraH2B-GFP* embryo at 4.0-4.5 dpc, *GFP* protein is located in the same cells as the native PDGFRa protein, and its expression also coincides with the location of other proteins typical of the primitive endoderm (Gata4, Gata6, Dab2). We also observed the same relationship in outgrowths from late blastocyst cultures for 72 hours in vitro.

The changes in the expression of the Pdgfr α H2B-GFP reporter are related to the stage of embryo development

Having confirmed that the PdgfrαH2B-GFP reporter line could serve as a marker for the primitive endoderm, we performed a detailed analysis of the location of the GFP protein in the embryos of this line (Hamilton et al., 2003). For this purpose, embryos were obtained at stages from 2.75 to 4.75 dpc every 2-3 hours, and live imaged using a confocal microscope. The presence of a weak GFP signal was found in some cells of the embryo at the morula stage, at the 16-32 cells stage, and at the 32-64 cell stage the signal was stronger and located both in external and internal cells. At the early blastocyst stage (29-63 cells, ~ 70-84 hours post coitum (hpc)), GFP was localized only in the ICM in about half of the embryos (18/30), while in the remaining ones - both in TE and ICM. At the mid- blastocyst stage (64 ~ 79 cells, ~ 87-96 hpc), GFP was located almost exclusively in the ICM, and the GFP-positive cell placement resembled the mosaic-like salt-and- pepper distribution previously described for other PrE markers (Chazaud et al. al., 2006, Gerbe et al., 2008), though it should be noted that in this case only one component is labeled, not two. In the majority of late blastocyst embryos (> 80 cells, ~ 96-114 hpc), GFP-positive cells were located only in the PrE layer adjacent to the blastocyst cavity. We also found that in embryos of the reporter line at the early postimplantetion stages (5.5 dpc), GFP is located in PrE derivatives, i.e. the visceral endoderm (VE) and Parietal Endoderm (PE).
Reporter expression analysis of PDGFR-H2BGFP therefore allowed us to determine the existence of several distinct phases of the pre-implantation embryo development. Because the time to obtain embryos after fertilization was not clearly correlated with the development stage, both morphologically and in terms of reporter expression, we applied the method of determining the stage by means of the number of cells. This method was first applied consistently to mouse embryos at the blastocyst stage in this publication, and has since been widely used by other researchers.

The use of reporter lines in combination with the confocal microscopy allows analysis of the cell fate and behaviour in live embryos cultured in vitro. The use of the PdgfraH2BGFP reporter line allowed us to investigate the fate of PrE cells in embryos grown in vitro and imaged using time-lapse movies. In embryos imaged every 15 minutes, we observed the same stages as in our earlier analysis of embryos isolated *ex vivo* every 2-3 hours, so *in vitro* culture and confocal imaging did not affect their development. The lack of negative impact on embryonic development was additionally confirmed by the analysis of morphology and expression of other

markers in embryos fixed after 17h of culture with imaging, as well as by transfer of such embryos to surrogate mothers, which resulted in the normal development to term and birth of healthy animals.

Detailed analysis of the obtained films allowed us to state that the transition from the stage in which the embryos show random, mosaic (salt-and-pepper) distribution of PrE cells, to the stage in which we can distinguish one distinct layer of PrE (GFP-positive), happen very quickly, often in just 15 minutes. After sorting into PrE layer occured, individual GFP-positive cells sometimes remained in the deeper (further from the blastocyst cavity) ICM layers, but in late blastocysts (over 128 cells) GFP-positive cells were no longer visible outside the PrE layer, in which the level of GFP fluorescence increased at the same time at this time.

Directional change of location and apoptosis of GFP-positive cells contributes to the sorting of cells during the formation of the PrE layer

Earlier publications suggest that the sorting of PrE and EPI precursors leads to the formation of spatially separated primitive endoderm and epiblast lineages (Rossant et al., 2003; Chazaud et al., 2006). To study this process, we analyzed the behaviour of 90 GFP-positive cells in six mouse embryos during the transition from early to mid blastocyst (32 to 64 cells) and 150 embryos during the transition from mid to late blastocyst (64 to 128 cells). We marked the starting position of each cell as (a) in contact with the blastocyst cavity (b) being in the deeper ICM layers. Then we marked the position of the cell (or daughter cells if they divided) in the same way after the movie ended. Almost half of the GFP-positive cells in the early / mid blastocysts (42/90) and 1/3 cells in the late stage blastocysts (52/150) finally reached a position other than the one they occupied at the beginning of the film (or other position) which occupied parental cells, if they divided).

In early-stage films, the majority of GFP-positive cells that initially were in the layer in contact with the blastocyst cavity maintained this position (72.5%, n = 40) and GFP-positive cells located in layers away from the blastocyst cavity only in 34% (n = 47) maintained their position (it should be noted that at this early stage the final sorting of the PrE layer is not yet achieved). This means that the movement of PrE precursor cells can take place in any direction, but the movement towards the blastocyst cavity and thus their final position prevails.

At a later stage (stage 64-128 cells), all GFP-positive cells in contact with the blastocyst cavity (100%, n = 64) maintained their position, whereas in the case of cells in the deeper ICM layers it

was only 15.8% (n = 57) . After clearly forming the PrE layer, the cells remaining in the deeper ICM layers downregulated GFP expression or undergo apoptosis.

We also found that embryonic cells before the average blastocyst stage (less than 64-cells) do not undergo apoptosis - in earlier embryos we did not find any one apoptotic cell (n=90), while in later stages the number of cells undergoing apoptosis increased with the total number of cells of the embryo. In addition, we observed that the frequency of apoptosis in GFP-positive cells in the deeper ICM layers was higher than in the layer in contact with the blastocyst cavity (48.2%, *n* = 27 Vs 8.1%, *n* = 123 cells). The observation of apoptosis in live embryos was made possible by the fact that the H2BGFP fusion protein is localised to chromatin, and effectively exposes the fragmentation of the cell nucleus associated with apoptosis. Life observations were then confirmed by TUNEL and immunofluorescence staining against active caspase-3. Apoptosis can be an additional mechanism eliminating those cells that are not in the right location, and contribute to the final sorting of the PrE and EPI layers.

We observed and distinguished different types of GFP-positive cell behaviour , and then we determined the percentage of each of them in the formation of the primitive endoderm. Our research indicates that the change in the position of cells in the ICM during the sorting of the PrE and EPI lineages may result mainly from the passive movement of cells caused by, e.g. by increasing the size of the blastocyst cavity and changes in the cell layout resulting from their division.

The expression of transcription factors specific for the PrE and EPI lineages is not mutually exclusive at the morula stage

To clarify the importance of the early expression of Pdgfra H2B-GFP, we examined the location of proteins associated with epiblast lineages and primitive endoderm. *Gata6* expression was previously described in murine blastocysts (Chazaud et al., 2006, Koutsourakis et al., 1999), as well as at late morula stage (Rossant et al., 2003), whereas NANOG expression was already described at the 8-cell stage (Dietrich and Hiiragi 2007). We also investigated the location of the transcription factor GATA4, whose co-expression with other PrE factors was demonstrated by microarray analysis (Kurimoto et al., 2006). In order to determine the age (developmental stage) of embryos, we used the total number of embryo cells, because our observations showed that

embryos obtained at the same time after fertilization, even from the same litter, differ in the number of cells and the expression profile of key transcription factors.

We have found the presence of GATA6 in the majority of embryos at the stage of 8 cells (9/11) and in some cells in 4-cell embryos (2/9). This is the earliest expression found for any PrE specific marker. In line with previous studies (Dietrich and Hiiragi, 2007), we found the presence of NANOG protein in the majority of 8-cell embryos (10/11). We detected GFP expression at the 16-cell stage in a some of the PdgfraH2BGFP embryonic cell, where it coincided with the expression of GATA6 from the beginning (Figure 3A). At morula stage, NANOG (n = 33) and GATA6 (n = 12) were detectable in almost all non-dividing inside and eutside cells (88.8% 770 cells were NANOG-positive, and 92.2% from 380 cells were GATA6 -positive), in line with previous observations (Strumpf et al., 2005). Interestingly, the location of these two factors at these early stages overlapped, and was not mutually exclusive. A similar expression pattern of these two factors occurred at the early blastocyst stage (up to 33 cells). Earlier studies presented only the location of these factors in salt-and-pepper manner at later stages of blastocyst development (Chazaud et al., 2006; Gerbe et al., 2008), and this is the first report presenting the coexistence of these factors in the same cells at earlier stages of development. To study the correlation of GATA6 and NANOG expression in the nuclei of individual cells, we calculated correlation coefficients. In embryos with a cell number less than 30, we did not find a correlation between the expression of GATA6 and Nanog. As the number of cells increased, the frequency of positive correlation increased, if all embryo cells were considered. However, when we performed the calculations only for the cells with the strongest signal (about 25% of the cells), we detected a strong negative correlation that increased with the number of cells.

Thus, it can be concluded that a positive correlation in cells with a low expression level of the studied factors may result from an increase in the level of expression in all cells at this stage, while negative correlation in cells with a high level of expression may suggest that the effect of mutual inhibition of PrE and EPi differentiation programs increases along with an increase in the expression level of these factors.

Changes in gene expression during the transition from 32 to 64 cells

We observed a decreasing share of NANOG- and GATA6-positive cells (63.3% of 2303 cells obtained in 54 embryos) and 62.7% of 346 cells, respectively, n = 8 embryos) in blastocysts that started the next cycle of cell division (from 33 to 63 cells). During this stage, the GATA6 and NANOG expression pattern indicated their ever stronger mutual exclusion (n = 8). In blastocysts over 36 cells, NANOG expression was no longer observed in all cells of the embryo.

The onset of GATA4 expression was observed in embryos that had at least 58 cells. We found the presence of this factor in 60% (n = 15) of embryos with 58-63 cells. The number of GATA4-positive cells in the embryos at this stage was variable (2-13 cells). Interestingly, we also found that unlike GATA6, GATA4-positive cells rarely co-expressed NANOG (only 15% of GATA4-positive cells were also NANOG-positive (n = 45), while 48.5% of GATA6-positive cells were NANOG- positive (n = 268).

At the 64-cell stage, a dramatic change occurred in the location of specific factors. The number of embryos in which GATA4-positive cells were found increased significantly between the 64- and 80-cell stages (81.5%, n = 27). In line with previous observations of other factors typical of PrE (Chazaud et al., 2006; Gerbe et al., 2008), GATA4-positive cells were arranged in a apparently random, mosaic pattern in the ICM. Similarly to the changes between the 32- and 64-cell stages, we observed only a small co-occurrence of GATA4 and NANOG signal in embryonic cells at 58-79 – cell stage (7.8%). It can therefore be concluded that GATA4 is a more specific marker of the PrE line than GATA6. Therefore, in the further stages of our research, we mainly used the GATA4 factor to label the PrE line.

About half of the embryos in the 80-100 cell stage showed partial or complete segregation of the GATA4-positive cells to the PrE layer, and NANOG-positive cells to the EPI layer (53.1%; *n* = 32), while the remaining cells were still salt- and-pepper -like. However, even in these unsorted cells, the expression of GATA4 and GFP (Pdgfra) almost never coexisted with NANOG expression. In embryos in which PrE was partially segregated (i.e. in which the PrE layer was visible but the single GATA4 positive cells remained in the deeper ICM layers), the GFP-positive cells in the deeper layers were characterized by a lower level of fluorescence. In 80.2% of embryos with a cell number> 100 (n = 96), GATA4-positive cells were found only in

the PrE layer, and in embryos with a cell number> 155 (n = 8), the formation of a uniform layer of PrE was completed.

We also found that the number of GFP-positive cells increased more or less linearly. Importantly, in PdgfrH2BGFP embryos with a cell number> 64, Gata4 was only expressed in GFP-positive cells, although GFP-positive and GATA4-negative cells were still present at this stage. This suggests that PrE precursor cells are recruited from the Pdgfra positive population at the 64-cell stage.

Taken together, this is the first study that presents analisys of the early events preciding PrE and EPI precursor emergence, and the first one that uses live imaging to uncover cell behaviours driving cell fate choice in preimplantation mouse embryo.

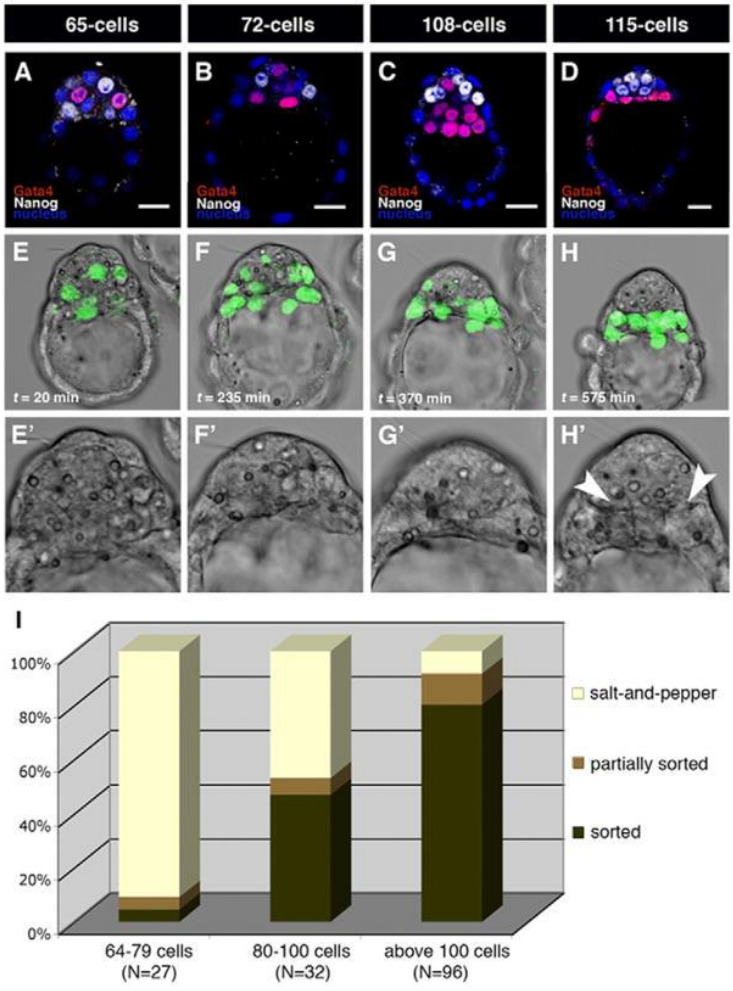


Fig. 3. A-D immunocolocalization fo NANOG and GATA4; E-H PrE sorting progression visualised by PdgfraH2B-GFP embryos; I – cell sorting progression in embryos with increasing numer of cells

2. **Piliszek, A.** , Kwon, GS and Hadjantonakis, A.-K. (2011) Ex utero culture and live imaging of mouse embryos. In: Vertebrate Embryogenesis: Embryological, Cellular and Genetic Methods. **Methods in Molecular Biology** . 770: 243-57, Ed. FJ Pelegri. Ed. Springer (book publishing, no IF), citations = 13

The use of a fluorescent marker in conjunction with confocal live imaging allowed us to discover previously unknown aspects of the formation of the primitive endoderm lineage in mouse embryos. A detailed description of the methods used, as well as other aspects of imaging the development of pre-and post-implantation embryos, we described in the chapter **"Ex utero culture and live imaging of mouse embryos" (Piliszek et al., 2011)** published in the book series: Methods in Molecular Biology.

Genetic engineering combined with new life imaging tools can revolutionize our current understanding of mammalian biology. The availability of many different fluorescent proteins provides the necessary tools for visualizing cells in living organisms. Characterization and cloning of Green Fluorescent Protein (GFP), originally from the organism of jellyfish Aequorea victoria, was awarded the Nobel Prize in the field of chemistry in 2008. Discovery and popularization of fluorescent proteins in combination with the possibilities of modern mouse genetics give us attractive tools for tracking cells of living organisms. It is now possible to create genetically modified strains of mice expressing fluorescent proteins in a manner specific to a particular tissue or cell lineage (reporter proteins). The use of such strains allows the imaging of dynamic cell behaviour in the context of a live embryo. Because mouse embryos develop in the uterus, experiments involving live imaging require *in vitro* culture conditions that closely mimic those *in vivo* . In recent years, significant advances have been made in the development of culture conditions for mammalian embryos in pre- and post-implantation stages. In the case of live imaging, it is important to combine the conditions that allow the development of the embryo (closely resembling those in the uterus), with the configuration of the equipment ensuring the best image quality. This can be achieved by using an appropriate environmental chamber integrated with the inverted microscope. The chamber provides the required stable temperature and gas concentration in embryo culture. In this chapter, we have discussed in detail the methods of breeding *ex utero* of mouse embryos in the preimplantation and postimplantation phases. In

particular, we described methods of isolation of embryos at various stages, culture conditions enabling live imaging and the use of confocal laser microscopy for vital visualization of processes occurring in the embryos of mice expressing fluorescent reporters, including nutrient composition, and gas mixes in subsequent development stages, hardware requirements and the best practices regarding the preparation of embryo cultures for imaging. Confocal laser microscopy excludes the signal from outside the focal plane, thanks to which it is possible to divide the sample optically, which can then be reconstructed in the form of a 3D image using appropriate software.

Expression of cell lineage-specific fluorescent reporters is an invaluable tool for studying mouse development in both wild-type and mutant embryos, in particular allowing the observation of *in-situ* gene expression *in* real time. In the last 100 years, mouse genetics has been developed into a powerful system of understanding mammalian biology at the molecular level. The mouse is a perfect model organism for testing mammalian biology due to its short duration of pregnancy, large litter size, small body size and resistance to infection. Unlike many other model organisms, such as zebrafish (*Danio rerio*) and *Xenopus laevis*, whose embryos are readily available for live imaging during normal development, mouse embryos develop inside the mother's body, which means that it is necessary to exactly mimic the conditions in the uterus during the *ex utero* breeding . The set of methods presented by us in this chapter allows to achieve conditions enabling the development of mouse embryos in vitro and their simultaneous imaging.

Artus, J. *, **Piliszek, A. *,** Hadjantonakis, A.-K. (2011) The primitive endoderm lineage of the mouse blastocyst: the sequential transcription factor and the regulation of differentiation by Sox17.

Developmental Biology 350 (2): 393-404.

MNiSzW item = 35, IF = 4.069, citation = 106

Continuing the analysis of the mechanisms of differentiation of the primitive endoderm line, in the next publication (Artus et al., 2011), we analyzed the expression profile and the role of transcription factors SOX17 and SOX7.

As in the previously published work of other researchers (Niakan et al., 2010; Morris et al., 2010), we found that SOX17 appears for the first time in preimplantation mouse embryos around stage 3.5 dpc. Thanks to a detailed analysis of the pre- and perimplantation stages, we have found that the SOX17 protein appears for the first time in the nucleus of the ICM cells at the stage of 32-64 cells, and thus at the stage that we have previously described as co-expressing the PrE (GATA6) and EPI (NANOG) markers. However, SOX17 is detected at this stage in only a small number (1-3) of cells. We also compared the localisation of SOX17 expressing the PdgfraH2B-GFP marker, the utility of which as a PrE marker was described in detail in our previous work (Plusa et al., 2008). SOX17 from the earliest moments in which it was detected, was in a subgroup of cells, which expressed GFP (not all GFP-positive cells were SOX17-positive, but all SOX17-positive cells were PDGFRa-positive). In the > 64-cell stage corresponding to the PrE and EPI distribution of the salt-and-pepper, the number of SOX17 positive cells increased and the cells were in complete overlap with the expression of PdgfraH2B-GFP and SOX17. At the late blastocyst stage (4.5 dpc), the SOX17 protein was located only in the PrE cells located on the the blastocyst cavity. Because in our previous work (Plusa et al., 2008), we showed that the expression of GATA4 begins when the salt-and-pepper pattern becomes apparent, ie at the 64-cell stage, and the expression of GATA6 starts already at the morula stage,thus, it can be concluded that the sequence of activation of particular factors associated with the differentiation of the PrE lineage is as follows: GATA6> PDGFRα> SOX17> GATA4.

Next, we examined the location of SOX17 protein in **PrE** derivative lineages, i.e. VE and PE lineages, at early postimplantation stages (5.25-5.75 dpc). Initially (5.25 dpc), SOX17 was detected in all VE cells, but later the signal in the distal part, i.e. covering the embryonic ectoderm, i.e. emVE (embryonic VE, Mesnard et al., 2006), was silent while remaining at a high level in part exVE (Extraembryonic VE), or VE covering the extra-embryonic ektoderm. The level of fluorescence in PE remained high in all cells.

Phylogenetic analysis of the SOX gene group showed that *Sox17* is in the F group together with the transcription factors SOX7 and SOX18 (Bowles et al., 2000). The sequence of the *Sox7* gene shows high homology with the *Sox17* sequence, however, previously it was not detected at preimplantation stages. Previous studies have indicated that *Sox7* is expressed in 7.5 dpc PE cells (Murakami et al., 2004), and that it plays a role in the differentiation of F9 cells towards PE *in vitro* (Futaki et al., 2004). Our analysis of the localisation of SOX7 protein at preimplantation

stages showed that SOX7 is a factor specifically associated with the PrE lineage in mouse embryos. SOX7 protein was detected at first in embryos on the stage above 64 cells, in a small subset of cells (1-3 cells in the ICM, average of 2.3 at the stage of 65-89 cells, n = 7). It was located only in ICM cells that were PdgfraH2BGFP-positive (in reporter embryos). We additionally found that SOX7 is located only in those cells that are GFP- positive, and at the same time are located on the edge of the ICM, in contact with the blastocyst cavity. Cells in deeper ICM layers, or GFP-negative cells that were in the outer ICM layer, did not express SOX7. It was expressed later than other PrE-specific factors (SOX17, GATA4). In embryos at stage 4.5 dpc, when the PrE layer was already completely sorted, SOX7 was located in all PrE cells, like other factors typical of PrE (GATA4, SOX17, PDGFRA, GATA6). Its late expression and position of SOX7-positive cells suggest that this factor is expressed only in PrE cells that have already obtained their final position in the PrE layer, and it is therefore the first known marker of cells already sorted and determined, and indicates that they differ in terms of gene expression from other precursor cells that have not yet been sorted. SOX7 may act as a positional signal amplifier, such as the apical DAB2 and LRP2 localization in some cells at the edge of the ICM (Gerbe et al., 2008), and allowing the final differentiation of the PrE cells. It is therefore the first known marker for cells already sorted and determined, and indicates that they differ in terms of gene expression from other precursor cells that have not yet been sorted out. Analysis of SOX7 localization in early embryonic germicidal cylinders (5.5 dpc) also showed that SOX7, like SOX17, is located in both PE and VE

The localization of SOX7 protein only in differentiated PrE cells in which the SOX17 protein was already present at earlier stages could suggest that the expression of SOX7 is dependent on SOX17. To verify this hypothesis, we examined the expression of SOX7 in genetically modified embryos lacking *Sox17* .

For this purpose, we used existing mouse lines with a conditional deletion of the *Sox17* Sox17cKO / cKO gene (Kim et al., 2007). To obtain KO / + animals, the cKO animals were crossed with the Sox2 :: Cre line (Hayashi et al., 2002), causing the deletion of SOX17 in ICM cells, and with the ZP3 :: Cre line (Lewandoski et al., 1997) causing deletion in oocytes, and thus allowing the removal of maternal *Sox17* transcripts (mzKO).

We found that the *Sox17* deletion in *zKO* embryos or in the embryos of the mzKO embryos did not affect the expression of the *Sox7* gene , because the SOX7 protein was found in PrE, as in

wild-type embryos. We have therefore confirmed that the expression of SOX7 is not dependent on the expression of *Sox17* . Moreover, in the *Sox17* - / - embryos, the PrE lineage is sorted properly. Perhaps SOX17 compensation by SOX7 occurs. Previous publications have shown that in *Sox17* - / - embryos at the stage of 8.0 dpc, the level of *Sox7* expression is significantly increased (Shimoda et al., 2007).

To learn about the roles of SOX17 in endoderm differentiation, we examined the differentiation in particular lineages in *Sox17* - / - embryos compared to *Sox17* +/- embryos and wild type embryos at the same stage of development. The embryos of all groups were morphologically indistinguishable, but their genotype was confirmed by genotyping (PCR). On stage 4.5 dpc seeds *Sox17* - / - they did not show the presence of SOX17 protein (as predicted), but the location of GATA4 in PrE and NANOG in EPI was unaffected, and both lineages were properly sorted. 5.5 dpc embryos were also morphologically correct. We also did not find statistically significant differences in the number of EPI and PrE cells in mutants compared to wild-type embryos. It can therefore be concluded that the differentiation and sorting of PrE and Epi lineages in mouse embryos is not dependent on SOX17.

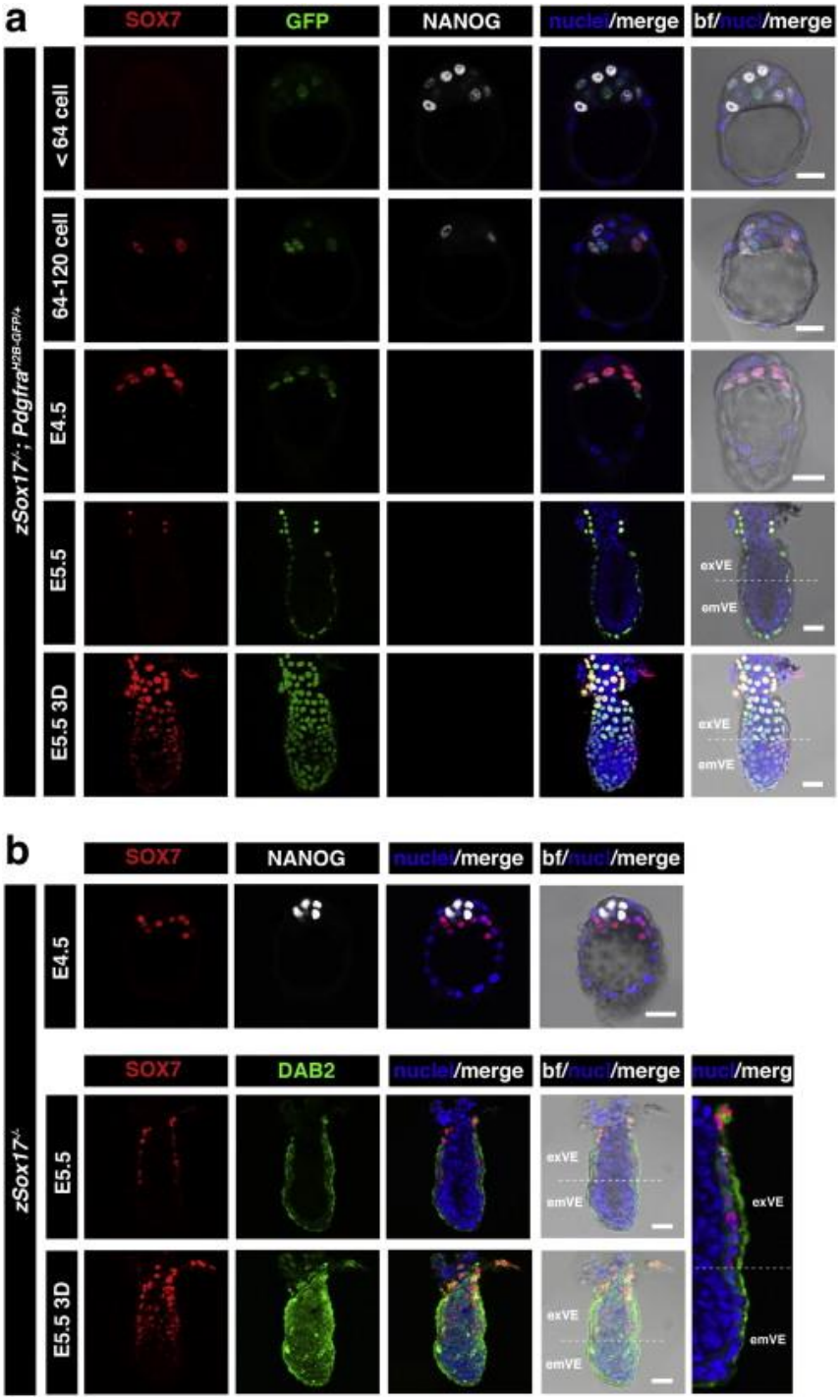


Fig. 4 Localisation of SOX7, PdgfraH2BGFP and NANOG in mouse mebryo at pre-and perimplantation stages

Piliszek, A. # , Madeja, Z., Plus, B. # (2017) Suppression of Erk signalling abolishes primitive endoderm formation does not promote pluripotency in rabbit embryo . **Development** 144 (20): 3719-3730.

corresponding author

MNiSzW points = 40, IF = 5.413, citations = 10

Introduction

Our knowledge about life processes is increased to a large extent thanks to research using model organisms. In mammalian development biology (as in many other areas of biology) the mouse (*Mus musculus*) has been successfully used as the main model organism for many years. Most basic research in experimental embryology is based on the mouse model, and hence most of our knowledge about the development of embryonic mammals. In my earlier studies (items 1, 2 and 3 of scientific achievement) I carried out research on the differentiation of primitive and epiblast endoderm on the mouse model. However, research on early embryonic development of mammals indicates that some of the observed processes, including the differentiation of the first embryonic lineages, may occur differently in rodents (mouse, rat) than in other mammals, including cattle (Berg et al., 2011) and primate (Bosze and Houdebine, 2010).

In order to get a more complete picture of the development of embryonic mammals, I undertook the analysis of the differentiation of primitive endoderm and epiblast in rabbit embryos (Oryctolagus cuniculus). The research was entirely financed from the my grant NCN SONATA (No. 2011/03 / D / NZ3 / 03992). The rabbit was chosen as the object of my research, because it is a model organism currently used in various biomedical research, including studies on lipid metabolism, atherosclerosis, diabetes, and cardiovascular diseases (Bosze and Houdebine, 2010) . It is also a convenient candidate for research in the field of development, due to its relatively small size (compared to livestock), short gestation period (31 days) and the possibility of obtaining embryos at a specific stage and embryo culture *in vitro* (Püschel et al. , 2010) . Phylogenetic studies also suggest a close evolutionary relationship between *Leporidae* and primates (Allard et al., 1996; Graur et al., 1996) . The early stages of rabbit embryo development are more similar to the development of other mammals (including humans) than mice, among others in terms of embryo morphology, course of gastrulation, as well as mechanisms of inactivation of the X chromosome and others (Okamoto et al., 2011). Despite such a convenient

set of traits, the rabbit has so far not been widely used in the study of the differentiation of the first embryonic cell lineages, and my research is the first detailed description of the course of these processes in rabbit embryos.

RESULTS

The analysis of the differentiation of the first cell lineages in mammalian species other than the mouse has so far been carried out mainly on embryos obtained *in vitro* (literature review in: Piliszek et al., 2016). To obtain a faithful image of the preimplantation of the rabbit, we analyzed embryos obtained *ex vivo* as a result of natural mating and development. The embryos were obtained at appropriate stages and then fixed and subjected to immunocytochemical analysis. The blastocyst stage in the pre-implantation rabbit development includes embryos from 60 to 5000 cells, and includes stages that may not have a counterpart in well-understood mouse embryonic development. In order to be able to reliably compare successive stages of development with each other, we have developed and for the first time applied a new rabbit staging system, based on the total number of cells in which, inter alia, 6 stages are described at the blastocyst stage (stages VI to XI), corresponding to the number of divisions that the embryonic cells have undergone, and differing, e.g. by expression profile of transcription factors. The system is based on the total number of cells of the embryo, similar to the one introduced by us for mouse embryos in the publication of Plusa et al., 2008 (item 1 of scientific achievement). The stages we distinguish are presented in figure 5.

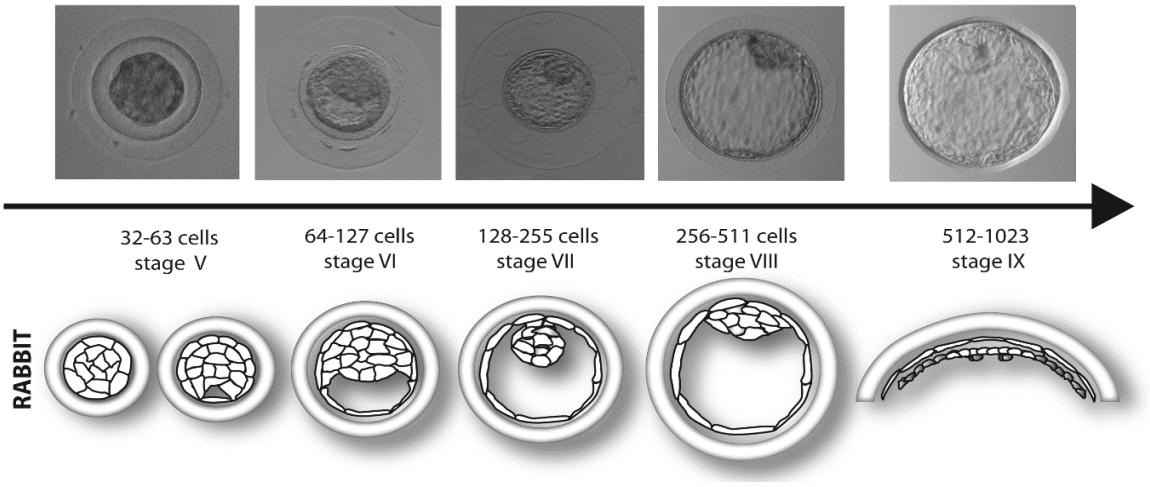


Fig. 5. Rabbit embryonic stage development system based on the total number of cells. The figure shows the stages during which the differentiation of primitive entoderm and epiblast takes place.

As a result of our preliminary research, we have found that the differentiation of PrE and EPI occurs in blastocysts consisting of ~ 100-1000 cells, i.e. in stages VI-IX (3-4 dpc). This work presents a detailed analysis of stages IV (morula) - IX (blastocyst).

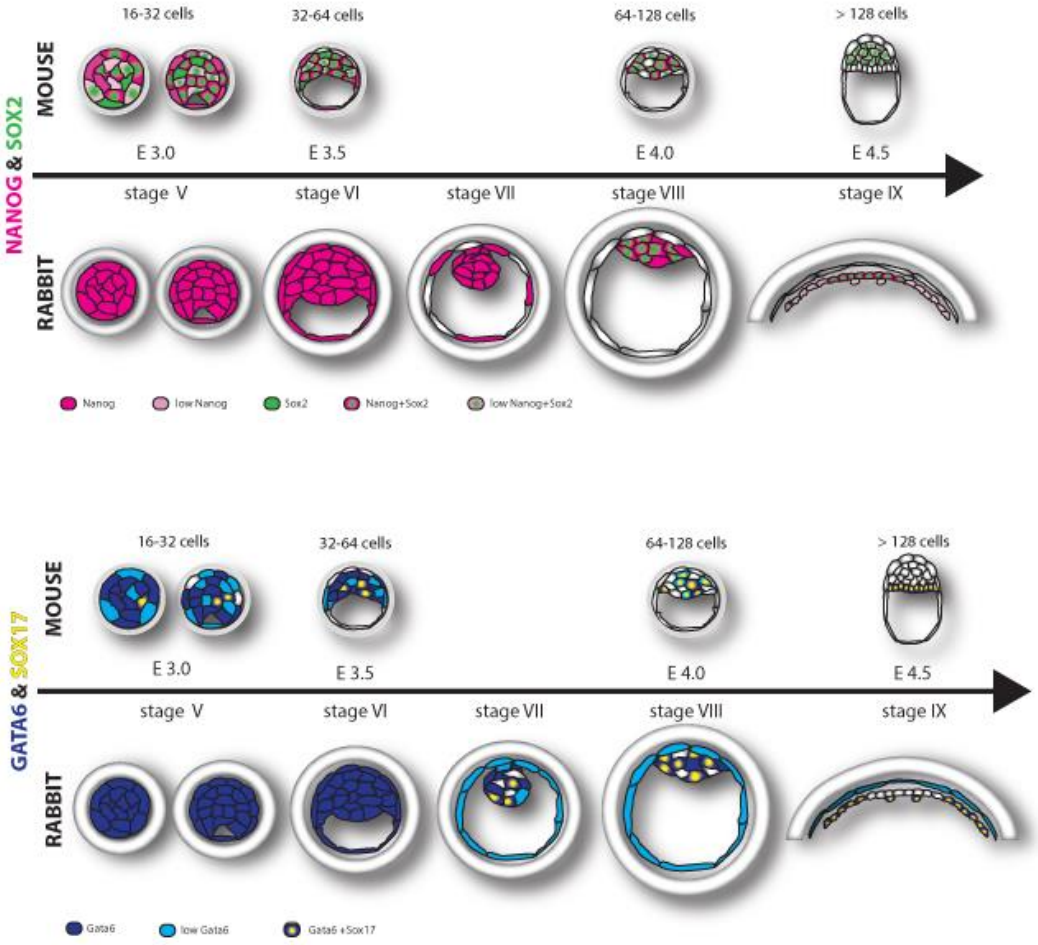
Determining the mutually exclusive expression of NANOG (EPI) and GATA6 (PrE) transcription factors is considered a key factor in the differentiation of EPI and PrE in mouse embryos (Chazaud and Yamanaka, 2016). Fixed rabbit embryos underwent immunocytochemical analysis to determine the presence of these key factors. We have found that early rabbit embryos (stage IV and V, morula, 3 dpc), similar to the mouse embryos (Plusa et al., 2008), express GATA6 and NANOG factors in all cells. In stages VII and VIII the majority of cells also co-expressed these factors, however, at these stages the first GATA6-negative / NANOG-positive cells appeared, which may suggest that at this stage the first stages of differentiation towards the epiblast occur. GATA6-negative cells accounted for stage VII 13.5% of ICM cells (n = 19), and stage VIII -33.9% of ICM cells (n = 21), however, all ICM cells remained NANOG-positive. Therefore, it can be concluded that the reduction of GATA6 expression in some ICM cells (probably EPI precursors) is not related to lowering NANOG levels in other cells. This is in

contradiction with the phenomenon observed in mouse embryos, where the mutual inhibition of GATA6 and NANOG is the main mechanism for the differentiation of EPI and PrE (Singh et al., 2007, Bessonnard et al., 2014). Downregulating level of NANOG expression in GATA6-positive cells in rabbit embryos was observed only at stage IX (Fig. 1E, E ') ; n = 7), when it was possible to see the first signs of sorting EPI and PrE into two separate compartments. Thus, the salt-and-pepper stage, characterized in the mouse embryos with the mutually exclusive expression of GATA6 and NANOG, is very short in rabbit embryos. We have also found that by sorting the EPI and PrE cells, the PrE layer does not form EPI at the border with the blastocyst cavity but forms a ring surrounding the epiblast. In conclusion, it should be noted that the mutual inhibition of NANOG and GATA6 transcription factors may not be involved in the initial stages of differentiation of EPI and PrE in rabbit embryos.

The GATA6 and NANOG factors in rabbit embryos are connected with the PrE and EPI lineages respectively, but the pattern of their expression does not indicate unambiguously that the initiation of the differentiation process is related to their expression. In the search for more specific markers, we decided to study the expression of SOX factors in rabbit embryos. In our previous work, we described the transcription factor SOX17, whose expression in mouse embryos is associated with the PrE lineage (Artus et al., 2011, item 3 of scientific achievement). SOX2, another transcription factor from the SOX group, is in turn associated in mice with EPI lineage differentiation (Wicklow et al., 2014). We found both of these factors in rabbit preimplantation embryos, but in later stages and in a smaller percentage of cells than NANOG and GATA6, which indicates that these are indeed more specific factors. SOX2 and SOX17 were not detected in the nucleus of the embryonic cells in most of the blastocysts of stage VII. Later, however, we found the location of these proteins in the ICM in a more reminiscent of the salt-and-pepper distribution. The number of SOX2 positive and SOX1-7 positive cells was similar in successive stages starting from VIII. Some of the cells co-expressed the two factors, which may indicate that they were not fully differentiated, however, in most ICM cells, the expression of SOX2 and SOX17 was mutually exclusive. In stage IX, the expression of these two factors was mutually exclusive in almost all cells, similar to what we observed earlier in the case of NANOG and GATA6 factors. Importantly, at this stage all SOX2-positive cells were NANOG-positive, and SOX17-positive cells were also GATA6-positive.Therefore, we can conclude that, similarly

to mice, SOX2 is involved in the rabbit embryos with the epiblast lineage and SOX17 - the PrE lineage.

A summary of the expression analysis of the studied transcription factors is shown in figure 6



Fig

6.Schematic representation of the expression profile of the most important transcription factors associated with the differentiation of epiblast and primitive entoderma in mouse and rabbit embryos

It has been shown that in mouse embryos, differentiation of PrE and EPI is dependent on the activity of the FGF signaling pathway / ERK (ang. **F** ibroblast **g** rowth **f** actor / MAP kinase (**m** itogen- **a** ctivated **P** rotein **K** InAs) (Kang et al., 2013 Krawchuk et al., 2013). In case when it is inactive, the entire ICM differentiates towards EPI. This was found, inter alia, by the

use of FGF / ERK pathway inhibitors - the culture of mouse embryos in their presence causes these embryos toform a morphologically correct blastocyst, which, however, is devoid completely the PrE lineage (Nichols et al., 2009). To study the effect of this signaling pathway on the differentiation of the first cell lineages in rabbit embryos, we blocked the ERK phosphorylation (and consequently the activation of the ERK signaling pathway) with the MEK inhibitor PD0325901at a concentration of 1 μM. The embryos were cultured *in vitro* from the morula stage (stage V) in RDH medium (Jin et al., 2000) with the addition of inhibitor, at 38.5 ° C, in a 5% CO2 atmosphere. In embryos from the control group, cultured *in vitro* under the same conditions, without the addition of an inhibitor, the presence of both SOX2 positive EPI cells and SOX17 positive PrE cells could be detected. In embryos in which ERK activity was blocked, no PrE cells (SOX17-positive) were found. It can therefore be concluded that, as in mouse embryos, the activity of the ERK signaling pathway is necessary for the differentiation of ICM cells towards PrE. Importantly, blocking this signaling pathway did not increase the percentage of SOX2 positive cells in the ICM (55.9%, n = 547 ICM cells in 13 embryos) compared to the control group (53.4%, n = 670 ICM cells in 9 embryos) also it did not affect the percentage of NANOG-positive cells. This is in contradiction with the data from mouse embryos, where the percentage of EPI cells increases at the expense of PrE cells, as all ICM cells differentiate towards EPI (Nichols et al., 2009, Chambers et al., 2003; Saiz et al., 2016). In rabbit embryos, however, we observed an increase in the percentage of ICM cells that did not express either SOX2 or SOX17, and therefore did not belong to the EPI or PrE lineages (control = 5.4% ERK inhibition = 44.1%).

This allows to conclude that ERK signaling pathway activity in rabbit embryos is necessary for proper differentiation towards the PrE , but its blocking is insufficient to induce differentiation towards EPI. (Figure 7)

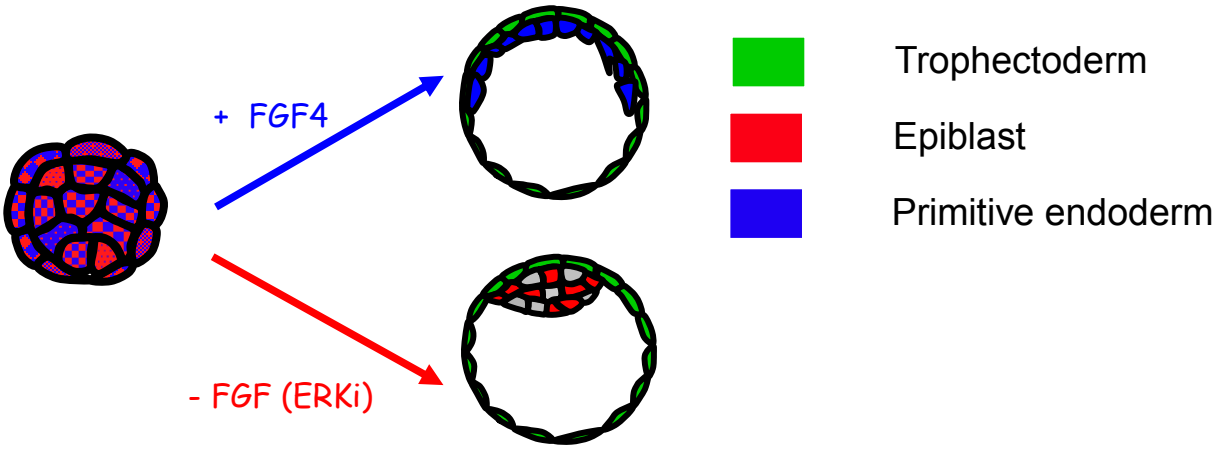


Figure 7. Schematic representation of the effect of blocking and activation of the MEK / ERK signaling pathway in rabbit embryos. Activation (+ FGF4) leads to the differentiation of all germinal node cells towards the primitive entoderma and their increased migration, while blocking the pathway blocks the expression of transcription factors associated with the primitive entoderm, without converting these cells towards the epiblast.

To better understand the effect of the FGF / ERK signaling pathway on the differentiation of rabbit embryonic cell lineages , we also subjected them to the activation of this pathway. The embryos were cultured under the same conditions in medium with FGF4 in concentration 100 ng / ml. Mouse embryos in these conditions create a morphologically correct blastocyst, which, however, is deprived of EPI cells. In rabbit embryos we also found no SOX2 positive EPI cells, but we also observed a change in ICM morphology. Namely, SOX17 positive cells were not arranged in a compact group similar to the ICM of the normal embryos, but formed a layer of cells that underlay the inner side of the TE. This behaviour of endoderm cells can be observed in the later stages of blastocyst development, when PrE differentiates into the VE lineage -

underlying ICM, and PE, which has the ability to migrate along the TE. It can therefore be concluded that activation of the FGF / ERK signaling pathway has resulted in the behaviour of cells consistent with the phenotype of PE cells.

The importance of research:

The research presented in this publication presents the first systematic analysis of the differentiation of the first cell lineages (epiblast, primitive entoderm) in rabbit embryos. Our results indicate a partially different mechanism of action of the MEK / ERK signaling pathway between rabbit and mouse embryos. They also confirm the similarities between rabbit and human embryos, and allow a wider use of the rabbit model in research on the early development of mammals.

Piliszek, A. #, Madeja, ZE (2018) Pre-implantation development of domestic animals. In: Cell Fate in Mammalian Development. **Current Topics in Developmental Biology** . Vol. 128, pp. 267-294; Ed. Hadjantonakis, A.-K., Plus, B. # correspondence author

A review entitled **Pre-implantation development of domestic animals (Piliszek and Madeja, 2018, habilitation achievement)** summarizes current knowledge about preimplantation development of farm animals, comparing it with more extensive knowledge gained through research on the development of mouse embryos. In particular, we present a systematic comparison of the timing of preimplantation development of many mammalian species, as well as detailed mechanisms of differentiation of the first cell lineages - trophectoderm, epiblast and primitive endoderm. This work can therefore be a summary of the research presented in this summary, comparing the development of the early stages of differentiation of both mouse and rabbit embryos, in comparison with the still limited knowledge about the development of other mammalian species.Understanding the molecular and cellular mechanisms of early embryonic development in non-rodent mammal species extends our knowledge of the basic mechanisms of differentiation and is necessary for the development of effective methods of assisted reproduction in agriculture, veterinary medicine and biomedical research

Key factors involved in the differentiation of TE, EPI and PrE, such as transcription factors and growth factors, may be common to different mammalian species, but their specific relationships vary depending on the species. Perhaps each species uses these elements in a different way, through differences in regulatory networks and the specificity of binding transcription factors (Kuijk et al., 2015) to adapt them to different needs, such as the size of the embryo, the type of implantation, and the length of the preimplantation period. In order to discover the mechanisms underlying the differentiation of the first mammalian cell lineages, and to distinguish between common elements and those characteristic of individual species, comparative studies of different models are necessary. Further understanding which aspects are common and which are divergent, will be essential for understanding early mammalian embryogenesis and differentiation mechanisms.

The research carried out by me on rabbit and mouse embryos, and presented in the publications included in the scientific achievement, are, I hope, a significant contribution to understanding the

mechanisms of embryonic development, in particular the differentiation of epiblast and primitive endoderm lineages

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5. Overview of other scientific and research achievements

During my doctoral studies at the Institute of Genetics and Animal Breeding of the Polish Academy of Sciences, I participated in a project led by Dr. AM Duszewska, in cooperation with prof. Z. Reklewski of the IGHZ PAN and prof. R. Słomski from the Institute of Human Genetics of the Polish Academy of Sciences. The aim of the research was to obtain transgenic calves after embryo transfer, into which the gene construct was introduced with the GFP reporter gene. I continued my cooperation with dr Duszewska in relations to the subject of breeding livestock embryos after graduating from doctoral studies. The result of the work carried out is the following publications:

1. Duszewska, AM, Wojdan, J., Gawron, W., Lipiński, D., **Piliszek, A.** , Wenta-Muchalska, E., Waś, B., Słomski, R., and Reklewski, Z. (2004) Obtaining calves after the transfer of embryos into which the gene structure has been introduced [Wettechnology] **.** 60 (12)
2. Duszewska, AM, Lipinski, D., **Piliszek, A.,** Słomski, R., Wojdan, J., Gawron, W., Wenta-Muchalska, E., Was, B., Chromik, A., Reklewski, Z. (2004) Cattle offspring after transfer of IVP GFP positive frozen / thawed embryos. **Animal Science Papers and Reports** 22: 603-610
3. Duszewska AM, Lipiński D., **Piliszek A** , Słomski R, Pławski A, Wojdan J, Gawron W, Juzwa W, Zeyland J, Wenta-Muchalska E, Reklewski Z. (2004) Controversial aspect of using GFP as a marker for the production of transgenic cattle. **Pol J Vet Sci.** 7 (4): 241-9.
4. Duszewska, AM, Rąpała, L., Trzeciak, P., Dąbrowski, S., **Piliszek, A.** (2012) Obtaining farm animal embryos in vitro. **Journal of Animal and Feed Sciences;** 21: 217-233 (Review Work)

The topic of my doctoral thesis was the developmental potential of somatic cells in mammals. I have shown that somatic cells (fibroblasts) introduced into the embryonic environment are able to divide and participate in embryonic development to the egg cylinder postimplantation stage. In connection with work on this subject, several reviews and chapters were also published. After completing my doctoral studies, I continued to work on this subject, including thecooperation with dr B. Płusa and dr K. Żyżyńska-Galeńska. The result of this

cooperation is, among others, demonstrating that the loss of the plasticity of ICM cells is not directly related to the differentiation towards PrE and Epi in undisturbed development.

5. Review work: Modliński, JA, **Piliszek, A.** , Karasiewicz, J. (2003) Development Potentials of Somatic Cells in Mammals [Developmental potential of mammalian somatic cells]. **Biotechnology** 2003 (1) 9-22
6. Review work: **Piliszek A.** (2004) Chimerowe zarodki ssakow: powstawanie i wykorzystanie [The production and use of mammalian chimeric embryos] **Biotechnology** 2004 (1) 142-155
7. **Piliszek, A.** , Modliński, JA, Pyśniak K., Karasiewicz, J. (2007) Foetal fibroblasts introduced to cleaving embryos contribute to full-term development. **Reproduction** 133 (1): 207-18.
8. Chapter in the monograph: Karasiewicz, J., **Piliszek, A.** , Sacharczuk, M., Modliński, JA (2008). Germinal mammalian somatic chimeras. In: **From the genome of tura to xenotransplantation** , Red. Z.Smorąg, R. Słomski, JA Modliński; OWN Poznań, 2008, p. 9-28.
9. Grabarek, JB, Żyżyńska, K., Saiz, N., **Piliszek, A.,** Frankenberg, S., Nichols, J., Hadjantonakis, A.-K., Płusa, B. (2012) Differential plasticity of epiblast and primitive endoderm precursors within the ICM of the early mouse embryo **Development** , Jan; 139 (1): 129-139
10. Review work: Żyżyńska-Galeńska, K. , **Piliszek, A.,** Modliński, JA (2017). From blastomeres to somatic cells: reflections on cell developmental potential in light of chimaera studies - a review. **Animal Science Papers and Reports** vol. 35 no. 3, 225-240.

Transgenic reporter lines are a great tool for analyzing the development of mammals. In cooperation with Dr. AK. Hadjantonakis I analyzed the TCF / Lef mouse line: H2B-GFP, which is a reporter for the Wnt / β-catenin signaling pathway. The use of fluorescent reporter lines has also been discussed in the review chapter, also prepared jointly by Dr. Hadjantonakis

11. Ferrer-Vaquer, A., **Piliszek, A** ., Tian, G., Aho, RJ, Dufort, D., Hadjantonakis, A.-K. (2010) A sensitive and bright single-cell resolution live imaging reporter of Wnt / ß- catenin signaling in the mouse. **BMC Developmental Biology** Dec 21; 10: 121.

12. Nowotschin, S., Garg, V., **Piliszek, A.** , Hadjantonakis, AK (2019) Ex utero culture and live imaging of mouse embryos. In: Vertebrate Embryogenesis. **Methods in Molecular Biology** 1920: 163-182, Ed. FJ Pelegri.

The visceral endoderm (VE) is one of the cell lineages originating directly from the primitive endoderm (PrE). The studies in which I participated showed that the BMP4 signaling pathway is able to induce epithelization of XEN cells (extraembryonic endoderm), resulting in a phenotype similar to extraembryonic VE.

13. Artus, J., Douvaras, P., **Piliszek, A. ,** Isern, J., Baron, MH, Hadjantonakis A.-K. (2012) BMP4 signalling directs primitive endoderm-derived XEN cells to an extraembryonic visceral endoderm identity. **Developmental Biology** , Jan 15; 361 (2): 245-62.

Another study conducted in the laboratory by Dr. Hadjantonakis presents the roles of Eomesodermin in the induction and migration of AVE (Anterior Visceral Endoderm). We have shown that the induction of AVE requires the activity of the *Eomes*, and is additionally associated with the activity of the *Lhx1* gene

14. Nowotschin, S., Costello, I., **Piliszek, A.** , Kwon, GS, Mao, CA, Klein, WH, Robertson, EJ, Hadjantonakis, AK (2013) The T-box transcription factor Eomesodermin is essential for AVE induction in the mouse embryo. **Genes & Development** 27 (9), 997-1002.

The main subject of my research, presented mostly in the main achievement of habilitation, is the differentiation of the primitive endoderm and epiblast lineages in mammalian embryos. In the work of Kang et al. 2013 (not included in the achievement), we presented an analysis of the role of FGF4 in the development of mouse embryos, by analyzing the development of *Fgf4* - / - embryos . The embryos of this line develop to the blastocyst stage, but are devoid of the PrE lineage, which indicates the requirement for FGF4 signaling in the development of PrE. In addition to my research work, I have also published several reviews on this subject

15. Kang, M., **Piliszek, A.** , Artus, J., Hadjantonakis, AK (2013) FGF4 is required for lineage restriction and salt-and-pepper distribution of primitive endoderm factors. **Development** 140 (2), 267-79.

16. Review work: Schrode, N., Xenopoulos, P., **Piliszek, A.** , Frankenberg, S., Plusa, B., Hadjantonakis, AK (2013) Anatomy of a blastocyst: cell behaviours driving cell fate choice and morphogenesis in the early mouse embryo. **Genesis** 51 (4), 219-33.

17. Review work: **Piliszek, A.,** Grabarek, J., Frankenberg, S., Plusa, B. (2016) Cell Fate in animal and human blastocysts. **Mol Hum Rep** 22 (10): 681-690.

18. Review work: Madeja, ZE, Pawlak, P., Piliszek, A (2019) Beyond the mice: non-rodent animal models for the study of early mammalian development and biomedical research. International Journal of Developmental Biology 63: 187 - 201

The work on developmental potencies of selectively enucleated zygotes is the result of cooperation with prof. JA. Modliński and dr. P. Gręda. We have shown that selectively enucleated zygotes can be recipients of nuclei even from 16-cell embryos. Such embryos are able to develop to term.

19. Greda, P., Modlinski, JA, **Piliszek, A.** (2015) Developmental potential of selectively enucleated mouse zygotes reconstituted with embryonic cell, embryonic stem cell and somatic cell nuclei. **Animal Science Papers and Reports** 33 (4), 323-336.

The work of Filimonow et al., 2019, was created in cooperation of many centers, including University of Warsaw and University of Manchester. The study allowed to show, among others, that changes in the expression level of E-cadherin may be related to the phenomenon of EMT (epithelial-mesenchymal transformation) in derivatives of the primitive endoderm (PE)

20. Filimonow, K., Saiz, N., Suwińska, A., Wyszomirski, T., Grabarek, JB, Ferretti, E., **Piliszek, A., Plusa** , B., Maleszewski, M. (2019) No evidence of involvement of E-cadherin in the product of bpococysts. **PLoS One** . 8; 14 (2): e0212109.