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Meeting Report

Mammalian Embryology Conference: celebrating the pioneering work of Andrzej K. Tarkowski

Warsaw, Poland, 25-26 October, 2013

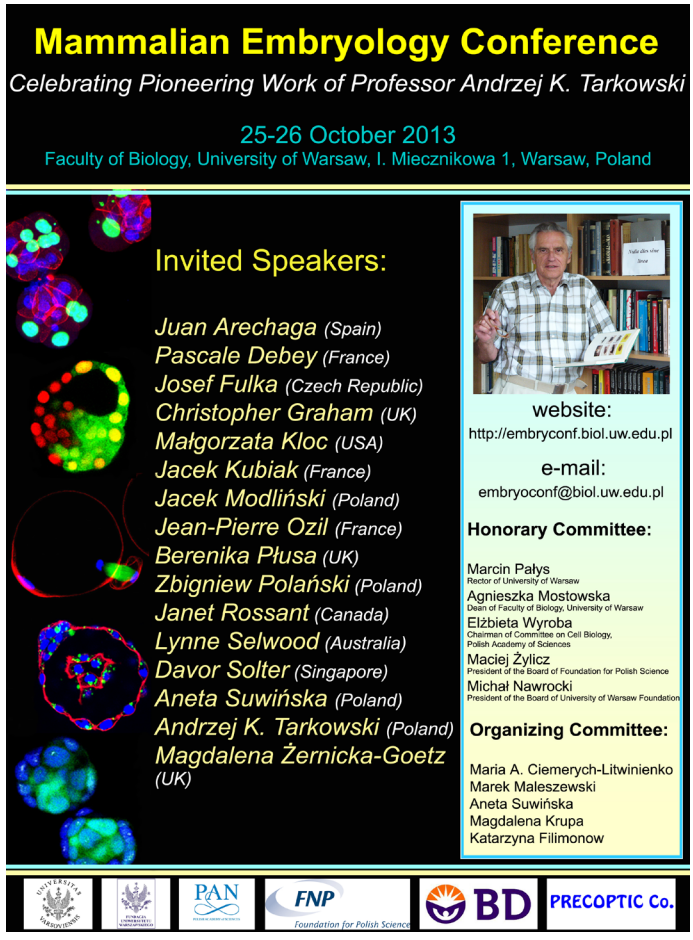
ABSTRACT During this Conference, a distinguished group of scientists from all over the world presented their latest studies on early mammalian development. Among them there were former students and colleagues of Professor Tarkowski. The Conference was opened with an address from the Dean of the Faculty of Biology, Professor Agnieszka Mostowska, who talked about the significance of the scientific and organizational activities of Professor Tarkowski, which, for the last 50 years, have been very important for Warsaw University. Sixteen lectures were presented over the two-days of the Conference. The topics covered studies of the regulation of the cell cycle during gametogenesis, activation of the oocyte at fertilization, control of cleavage, formation of the earliest cell lines of the embryo, cell totipotency, pluripotency, differentiation during early development, primordial germ cells, developmental epigenetics and evolutionary aspects of the study of development. Many of the topics presented had a beginning in the seminal works of Professor Tarkowski, who pioneered studies on the developmental potency of isolated blastomeres, on the formation of experimental chimaeras, on the artificial activation of oocytes and the development of parthenogenetic embryos. A detailed program of the Conference can be found at the following link: http://embryconf.biol.uw.edu.pl

KEY WORDS: *mammalian experimental embryology, embryo, blastomere potency, chimaera, parthenogenesis, stem cell, germ cell, gamete*

Introduction

The Faculty of Biology of the University of Warsaw held a two-day Mammalian Embryology Conference on October 25 and 26th, 2013 to celebrate the 80th birthday of Professor Andrzej Krzysztof Tarkowski, one of the pioneers of mammalian developmental biology. The conference was organized by his former students, and as invited speakers hosted leading scientist, many of whom Professor Tarkowski mentored in the past.

Professor Tarkowski started his career as a biologist in 1950 when he was admitted to study at the Faculty of Biology and Earth Sciences, University of Warsaw. In 1955 he graduated with a M.Sc. degree. Ever since, his career has remained linked to the University of Warsaw. In 1959 he received a Ph.D. and in 1963 a D.Sc. degree at the Faculty of Biology, University of Warsaw. In 1972 he was appointed professor, followed by full professorship in 1978. Between 1964 and 2003 Professor Tarkowski was the head of the Department of Embryology, and in 1972-81 and 1987–2003 he was the head of the Institute of Zoology in the Faculty of Biology, University of Warsaw. During his scientific career, Professor Tarkowski has collaborated with many research centres around



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the world. He was a Fellow of the Rockefeller Foundation in the Department of Zoology, University College of North Wales (UK), and served as a visiting professor of the Royal Society at the University of Oxford (UK), Rockefeller University of New York (USA), University of Adelaide (Australia), and Institute Jacques Monod CNRS and Paris University XVII (France). Professor Tarkowski is also a member of many academic institutions and societies, such as the Polish Academy of Sciences, the Polish Academy of Arts and Sciences, the French Academy of Sciences, the American Academy of Sciences and Academia Europaea. In 1997-2004 he was also a member of the Advisory Board of the Foundation for Polish Science. Scientific achievements of Professor Tarkowski were recognized with numerous prestigious scientific awards and honors, including the Albert Brachet Prize of the Royal Academy of Belgium (1980), the Polish National Award (1980), the Alfred Jurzykowski Foundation Award (1984), the Embryo Transfer Pioneer Award, the International Embryo Transfer Society Award (1991) and the Commander's Cross with Star of the Order of Polonia Restituta (2012). In 2002 he received the Japan Prize of the Science and Technology Foundation of Japan. He has been also awarded a Doctor Honoris Causa degree of the Jagiellonian University (2000) and of the Medical University of Lodz (2005).

In 2003 Professor Tarkowski retired. However, he still continues his research. He is characterized by passion, joy and great imagination in undertaking new experiments and inventing new techniques. Aside from his professional work, Professor Tarkowski is also passionate about photography. His photographic exhibitions – "Botanical Impressions", "Tree and Wood", "The Earth We Walk On" and "On the Border of Nature and Abstraction" – have been presented in many Polish cities.

Pioneering research of Professor Andrzej K. Tarkowski, initiated in the 1950s, has proved to be fundamental not only for modern mammalian developmental biology but also for progress in animal breeding and assisted reproduction. In 1959, Professor Tarkowski showed that a single blastomere isolated from a 2-cell stage mouse embryo is able to support full term development and that the resulting mouse is healthy and fertile. These experiments confirmed earlier studies in the rabbit and proved that preimplantation development of mammals is highly regulative. Furthermore, these findings contributed to the development of preimplantation genetic diagnosis, a technique used in *in vitro* fertilization (IVF) clinics throughout the world in which one blastomere of the preimplantation embryo is removed to provide material for genetic analysis to identify congenital defects in embryos before they implant. In 1966 Tarkowski also developed a technique for studying chromosomes in oocytes and early embryos, which has been widely used in early studies on IVF in humans.

In 1961, Tarkowski reported the birth of the first chimaeric mice produced experimentally by the aggregation of two genetically distinct cleaving embryos. Since then chimaeric animals, obtained in different ways, have become an important tool in the study of development and cell differentiation in mammals. The modified method is used to this day for immunological, genetic and oncological studies, and also for obtaining transgenic animals, offering one of the most powerful tools currently available for studying gene function in living animals.

In later years, Professor Tarkowski and his collaborators showed for the first time that artificially activated mouse oocytes can develop till mid-gestation. Together with his group, he also studied developmental effects of induced chromosome aberrations such as triploidy, tetraploidy, and also diploid/tetraploid and diploid/triploid mosaicism. Working with Jacek Kubiak, Professor Tarkowski devised a technique of blastomere electrofusion leading to the generation of tetraploid embryos. This technique together with Tarkowski's studies on oocyte maturation, fertilization and nucleo-cytoplasmic interactions in germ cells and early embryos, including remodeling of somatic nuclei transplanted to oocytes, has contributed substantially to the development of mammalian cloning and are also used during the generation of genetically modified animals.

Today, science still greatly benefits from the research initiated decades ago by Professor Tarkowski. It is very difficult, if not even impossible, to imagine the advances in mammalian developmental biology and also biomedical sciences that we have witnessed in recent years without his pioneering work in mammalian embryology.

Day 1

Chris Graham *(University of Oxford, UK)*

Andrzej Tarkowski and Chris Graham worked on the problem of nuclear transfer in mice from the late 1960's onwards, both separately and together encouraging and exhorting each other to explore nearly every possible way of making the recipient egg develop further. Making little progress in this endeavor, they promoted detailed studies of mammalian eggs.

Chris Graham discussed his current interest: the problems of describing & analyzing the speed and direction of movement of the mouse and human activated egg. The detailed information is from the mouse. Fast movements occur soon after egg activation and during these spasms there are abrupt shape changes in cell protrusions such as the emerging second polar body and the fertilization cone. These movements last until mature pronuclei are formed and they occur in synchrony with the peaks of free calcium ions in the cytoplasm. The apparent speed of these movements entirely depends on the frequency at which they are recorded or analyzed, so that an analysis interval of 120 secs displayed a mean speed at least 1/10 th of that at an interval of 1 sec. This discrepancy is partly explained by the fastest movements been confined to 1 sec or less. In addition, local movements around the fertilization cone are often twice the speed of the mean speed across the cytoplasm.

During the approach to the first mitotic cleavage, pronuclear envelope breakdown (PNEBD) occurs over an hour before cytokinesis. Around this time the wrinkled or distorted outline of the egg smoothes over as the egg rounds up. The egg then progresses through several cycles of irregular shape and round up before the final round up that precedes the elongation of mitotic anaphase. There are abrupt movements during this period, partly because the egg has lost volume and often rotates inside the zona, particularly when the egg rounds up. The slow cycles can most easily be perceived by collecting or analyzing these movements at long time intervals, for instance every 120 seconds. The analytical frequency must be matched to the phenomenon and no fixed frequency can capture all features of a movement.

The human egg also displays a variety of slower movements that have no obvious parallel in mice. These include the rotation of a granular state in the cytoplasm and the appearance of a halo, a cleared area of cytoplasm around the whole periphery of the

zygote. These currently appear to be slow movements.

The audience was the subject of an experiment that showed that a quarter has different higher brain functions from the rest. The result emphasized the problem of presenting visual information to a variety of perception mechanisms.

Jacek Modliński *(Institute of Genetics and Animal Breeding, Poland)*

Jacek A. Modliński worked in Andrzej K. Tarkowski's lab from 1966 till 1991 developing, under his supervision, many of the pioneering mouse nuclear transfer techniques. A.K. Tarkowski was the supervisor of his MSc and PhD Theses. In 1991 he moved to the Institute of Genetics and Animal Breeding Polish Academy of Sciences where he is a Professor and Head of the Department of Experimental Embryology. Recently one of his fields of interests are evolutional, epigenetical and molecular aspects of mammalian embryonic diapause and the long-term effects of ART. These studies were conducted with close collaboration with Dr Grażyna Ptak's group from Teramo University (Italy). Embryonic diapause (ED) is a temporary arrest of embryo development. This phenomenon is widespread both in plant and animals species as a response to specific situations when further embryonic development is imperiled due to – for example – harsh climates, temperature fluctuations, under-nutrition or lactation. In mammals, ED occurs at the blastocyst stage and is characterized by the delayed implantation of the blastocyst in the uterus. Although ED occurs only in less than 2% of mammalian this phenomenon has been observed in species that belongs to seven highly represented mammalian orders. It is not clear if ED occurs in *Primates* (including human) since its occurrence in this order was not explicitly investigated. It is commonly believed that the phenomenon of embryonic diapause evolved independently in different mammalian orders. However, Ptak's and Modliński's previous unpublished observations suggested that the ED might be an evolutionarily conserved phenomenon. If so, then it should be possible to induce ED in blastocysts of non-diapausing (ND) mammals such as livestock species. To prove this hypothesis, they tested whether blastocysts from domestic sheep which is ND species could enter into diapause following their transfer into pseudo-pregnant mouse in which uteri diapause conditions were experimentally induced by ovariectomy and progesterone treatment. After 7 days the uteri were flushed out and viable ovine blastocysts were recovered. The viability of these blastocysts was confirmed by their reexpansion within 1–2 hours of *in vitro* culture. None of ovine blastocysts had elongated (as normally observed in ruminants), and their size had not changed during the stay in mouse uteri. In contrary to the control groups represented by ovine blastocysts cultured *in vitro* and mouse blastocysts flushed from ND mice which exhibited high level of DNA synthesis, the diapausing, viable ovine and murine blastocysts showed arrested DNA replication as was assessed by BrdU incorporation. Also, the percentage of dead cells was significantly lower in diapausing ovine and murine blastocysts than in blastocysts from the controls groups.

Further analysis revealed differential expression of ED markers between diapausing and ND ovine blastocysts. In diapausing blastocysts, genes that positively regulate cell proliferation (PCNA) and signaling (HB-EGF) were down-regulated, whereas the anti-proliferative BTG1 gene was strongly up-regulated. CB1 which is normally down-regulated before implantation, was highly expressed in diapausing ovine blastocysts. This study showed, for the first time, that induction of diapause in blastocysts of ND species is fully reversible. Diapausing ovine embryos restarted growing *in vitro*, can hatch from *zonae pellucidae* and, after transfer to the recipient ewe, are able to develop to full-term at a proportion significantly higher than in control group. All offspring had also the normal birth weight and were healthy. Demonstration that ND ovine embryos can enter into diapause implies that ED is phylogenetically conserved phenomenon and not secondarily acquired by embryos of diapausing species and questions the current model of independent evolution of ED in different mammalian orders. Therefore, it provides a starting point to verify the flexible occurrence of ED in mammals and opens new perspectives for reproductive and evolutionary biology. If diapause does occur in all mammals, this has huge implications for human pregnancy. Based on this finding, it is important to verify whether diapause may occur also in human embryos.

Blastomere biopsy (BB) is an essential technique for performing preimplantation genetic diagnosis (PGD), a screening test that can detect genetic abnormalities of embryos before their transfer *in utero*. There is limited understanding of the post-natal consequences and safety of BB, so far. To check it the 8-cell stage mouse embryos were subjected to a single blastomere biopsy and then cultured *in vitro* until the blastocyst stage. Two control groups were created, one consisting of embryos cultured *in vitro* without any manipulation (IVC) and one of embryos developed entirely *in vivo* (*in vivo*). Embryos from all groups were transferred to pseudopregnant female mice at the blastocyst stage. Body growth parameters and developmental landmarks of the resulting offspring were observed during their entire lifespan. Furthermore, validated behavioral tests were used to assess early communicative functions, startle reflex, and anxiety- and depression-like behaviors. It was found that male mice derived from BB exhibited peculiar behavioral alterations and changes in body weight. BB-derived mice showed increased body weight with respect to controls as early as the second week of life. Adult males displayed decreased time of immobility in the tail suspension test and deficits of the habituation to – and prepulse inhibition of – the startle reflex. Communicative skills and anxiety-like responses were not affected by BB. Thes data demonstrate that long-term programming of postnatal development and behavior is affected by BB in mice, suggesting that PGD procedures could be a risk factor to late-onset, neurodevelopmental disease predisposition. Thus, in light of our observations, long-term follow-up in humans or other primates generated after BB is needed.

Jacek Kubiak *(University of Rennes, France)*

Jacek Kubiak trained first as a technician and then as a PhD student in Andrzej K. Tarkowski's lab in 80's of the XXth century. He then left for the postdoc to Bernard Maro's lab in Paris, and initiated his independent research in late 90's. His first scientific achievement, as a technician, was the adaptation of the method of electrofusion of mouse blastomeres and oocytes together with Andrzej Tarkowski further largely used for cloning experiments. Using this technique he became rapidly interested in cell-cycle regulating factors and during his PhD training he followed the behavior of nuclear lamins and microtubules in hybrids between mitotic and interphase mouse embryos. He also found that the meiotic cell cycle is programmed to repeat M-phases not twice, as usually observed during oocyte maturation, but in series of three or four. He began to work with Bernard Maro when the fundamental

molecular machinery regulating the cell cycle was described by Paul Nurse and Tim Hunt (CDK1 and cyclins). Fascinated by modifications of the cell cycle regulation in mouse oocytes and early embryos, he worked on cyclin B metabolism and showed that even in MII-arrested oocytes cyclin B undergoes rapid turnover indicating that the M-phase-arresting factor CSF does not block totally cyclin B degradation. This was further shown also in *Xenopus laevis* oocytes. Further work of Jacek Kubiak was focused on ERK1/2 MAP kinases and Mos pathway during mouse oocytes maturation and fertilization. As the biochemistry of cell cycle-regulating proteins is much easier using *Xenopus laevis* cell-free system than using small mouse oocytes and embryos, he turn to this model when moved to Rennes in Bretagne, West France. He has shown that the maternal Aurora A mRNA is deadenylated after fertilization by mRNA-binding protein EDEN-BP-dependent mechanism. Further, he has shown EDEN-BP is regulated by phosphorylation by CDK1 and ERK2. Thus, cell cycle-regulating proteins are regulated on the translational level by another set of cell-cycle regulators. Further, he has shown that CDK1 is inactivated when cyclin B dissociate from the kinase and before its proteolytic degradation. This opened a possibility that CDK1 inactivation may be mediated or accelerated by a specific inhibitor. In a proteomic screen dedicated to identify CDK1 partners his lab found that CDC6, a well known S-phase regulator, is associated with CDK1 upon M-phase. As data from yeast suggested that CDC6 may participate in M-phase exit, Kubiak's lab turned to study the potential role of CDC6 in CDK1 regulation during *Xenopus laevis* embryonic mitosis. This revealed that CDC6 indeed mediates CDK1 inhibition upon M-phase entry, progression and the exit in *Xenopus* embryo cell-free extracts. The comparison with CDC6 from mouse oocytes and embryos made in collaboration with dr. Ewa Borsuk, from Andrzej Tarkowski's lab, let them to conclude that CDC6 may act as CDK1 inhibitor also in mouse cells. These experiments are under progression in Rennes and in Warsaw.

Magdalena Żernicka-Goetz *(University of Cambridge, UK)*

Magdalena Żernicka-Goetz is a former PhD student of Prof. AK Tarkowski. Her group work aim to understand how regulative plasticity of early mouse embryos works: what is its mechanism of regulative development and how are the cell fate decisions taken? To address this she first had to develop new ways that would allow following cell fate in unperturbed development, when embryo does not regulate, in order to relate this to what happens when embryo has to recover from experimental manipulation. The development of variant approaches for following individual, non-invasively labeled cells led her and her group to set up the first, long-term time-lapse recording and computer assisted tracking of pre-implantation development that allowed tracking of all cells continuously as the cell fate becomes established. These studies revealed: First, that the early cell fate decisions are anticipated by unexpected heterogeneity in chromatin organization and behavior of transcription factors, such as Oct4 and Cdx2, between cells as early as at the 4-cell stage that bias cell fate. Second, the first wave of asymmetric divisions generate inside pluripotent epiblast cells, that will give rise to the body, and outside cells that are biased to give rise to extra-embryonic lineages: primitive endoderm and trophectoderm that have supportive and signaling roles. Third, subsequent waves of asymmetric division lead to segregation of these two extra-embryonic lineages - trophectoderm on the outside and primitive endoderm on the inside. Fourth, if cells are not positioned according to their origin and fate, they will swap their positions; those, which don't, either undertake apoptosis or switch fate. Together these and other studies led her group to propose that cell fate in the mouse embryo begins to be specified earlier than had been previously expected and neither randomly, as previously thought, nor deterministically. Instead cell fate becomes established gradually as heterogeneity between cells biases cell behavior and fate. This fresh view on first lineage specification has opened up many new questions and predictions and she discuss those that she is currently testing.

In contrast to pre-implantation stages, development of the embryo as it implants and shortly thereafter has been hidden from direct view and inaccessible to experimentation because it takes place within the body of the mother. Yet this specific period of development is critical: it is the time when the embryo transforms form a simple ball of three types of cell into the egg cylinder within which the anterior-posterior axis becomes established and the body will form. To gain direct insight into this developmental transition Prof. Zernicka-Goetz with her group has established a culture system and chemically defined medium that enables embryos to develop, be manipulated and imaged throughout implantation stages outside the mother. She discussed the new insights that this is bringing including a new hypothesis of how the blastocyst transforms into the egg cylinder.

Aneta Suwińska *(University of Warsaw, Poland)*

Aneta Suwinska obtained a M.Sc. degree in 2003 followed by a Ph.D. degree in 2008 in the Department of Embryology, University of Warsaw, Poland, both under the supervision of Prof. Andrzej K. Tarkowski. She then initiated her independent research aiming at addressing the one of the most fascinating questions in embryology: how and when blastomeres become committed to different cell lineages and what factors are responsible for the choice of cell fate.

During mouse embryogenesis initially indistinguishable embryonic cells (blastomeres) differentiate, specialize, and finally form distinct cell lineages: trophectoderm (TE) and, arising within the inner cell mass (ICM), epiblast (EPI) and primitive endoderm (PE). After implantation of the embryo EPI contributes to the tissues of the embryo body, PE gives rise to the endoderm layer of the yolk sac, and TE forms the placenta. As the development progresses blastomeres gradually restrict and lose their initial totipotency, i.e. the ability to give rise to both embryonic and extraembryonic lineages and become directed into a specific developmental path. Suwinska together with Tarkowski and colleagues aimed to unveil full range of developmental capabilities of blastomeres in artificially created circumstances: when normal development of embryos is experimentally disrupted.

Firstly, they showed that when sister inner and outer blastomeres of 16- and 32-cell embryos were disaggregated into single cells and reaggregated at random within the same embryo, the majority of cells relocated to their original position (outside or inside), and the aggregated embryos eventually developed into normal, fertile mice. Moreover, embryos constructed exclusively from inner or outer blastomeres of 16-cell embryo, but not of 32-cell embryo, were able to develop into fertile mice.

Secondly, they surrounded a single, diploid blastomere of 16- or

32-cell embryo with the tetraploid carrier blastomeres of different genotype. When they used individual 16-cell stage blastomeres to construct separate aggregate-embryos with tetraploid cells, this approach allowed them to generate genetically identical twins, triplets and quadruplets derived exclusively from the diploid 16-cell stage blastomeres. Blastomeres seem to lose their full developmental potential around the 32-cell stage. Authors were able to generate foetuses from single blastomeres isolated from this developmental stage only sporadically.

Thirdly, Suwinska's group wished to address the question of how and when epiblast *vs.* primitive endoderm specification takes place within the ICM and what mechanisms underlie allocation of these lineages. They verified the relationship between the time of generation of inner cells contributing to the ICM and their further differentiation. They devised a method of dual labeling of embryos to distinguish both generations of inner cells (derived from the first and the second wave of asymmetric cell divisions). Their results showed that the inner cells derived from the first and the second wave of asymmetric divisions had different patterns of gene expression. However, these differences did not restrict potencies of these cells. When aggregated into homogeneous aggregates inner cells of both generations developed successfully to normal blastocysts. Moreover, tracking the fate of cells in immunosurgically isolated ICMs Suwinska and collaborators confirmed that their flexibility, especially in regard to their differentiation into EPI or PE, was maintained for substantially long time. Although ICMs isolated from both early (64-cell) and late (containing over 80 cells) blastocysts were no longer able to differentiate into TE, they formed a layer of PE on their surface after several hours of *in vitro* culture. Applying two different methods of cell tracking they proved that even as late as in the expanded periimplantation blastocyst stage, ICM cells' fate was not irrevocably determined and cells were still able to change their destiny.

Day 2

Zbigniew Polański *(Jagiellonian University, Poland)*

Zbigniew Polański presented the studies related to the functionality of the Spindle Assembly Checkpoint (SAC) during mammalian female meiosis. The SAC is functional in many cell types and comprises a mechanism which enables errorless segregation of the chromosomes in dividing cells by blocking the onset of anaphase when sensing the spindle or chromosome anomalies. Reduced SAC activity during female meiosis may promote formation of aneuploid oocytes, thus leading to embryonic aneuploidy resulting in pregnancy loss or birth defects. On the other hand, Polański Lab in collaboration with other groups used the model of LT/Sv mice to show that the inability to switch SAC activity blocks progression of meiosis in the oocytes from this strain by arresting them at the first meiotic metaphase. This demonstrates that the SAC may contribute to the reproductive disorders not only through embryonic aneuploidy since its overregulation in LT/Sv oocytes blocks meiosis at metaphase and thus reduces fertility. A number of reports have indicated that arresting the oocyte meiosis at the metaphase I stage is indeed a clinically relevant problem related to the human fertility. Thus, the studies on the LT/Sv model show the potential link between SAC function and recurrent human oocyte metaphase arrest and may contribute to the elucidation of the detailed mechanism of such disorder. Polański and coworkers showed that the nucleus from the LT/Sv oocyte when transplanted into the cytoplasm of a wild type oocyte does not arrest at metaphase. These results indicate that transplantation of the nucleus from patients' oocyte into the healthy recipient oocyte cytoplasm might provide effective treatment in the causes of human infertility linked to oocyte metaphase arrest. Recent work by the group demonstrates that the frequency of the SAC associated metaphase I arrest in LT/Sv oocytes drops in older females. This suggests that maternal age-related aneuploidy in mammals may result from reduced effectiveness of the SAC in the oocytes of older females.

Joseph Fulka Jr. *(Institute of Animal Science, Czech Republic)*

Josef Fulka, Jr. presented results of studies oriented on the role of nucleoli in mammalian oocytes and early embryos. Mammalian fully grown immature (GV stage) oocytes and early-cleavage stage embryos contain atypical nucleoli that are morphologically distinct from nucleoli that can be found in advanced-cleavage stage embryos, embryonic stem cells or in differentiated cells. In the first case, nucleoli contain only fibrillar material, whilst in the second case nucleoli contain fibrillar, dense fibrillar and granular material. Nucleoli in somatic cells are relatively well characterized (contain several thousands of different proteins) and are involved in cell cycle regulation, chromosome segregation, cell differentiation and so on. On the other hand, nucleoli in fully grown oocytes and early embryos are only poorly characterized. Interestingly, nucleoli in growing oocytes are morphologically similar to differentiated cell nucleoli but their morphology gradually changes with the oocyte growth. It must be noted here, that these changes occurs in interphase-like cells (intact membrane, no intervening M-phases). As the embryo develops, nucleoli gradually change their morphology and in morula-blastocyst stage they are morphologically similar to nucleoli in differentiated cells. Thus, it has been suggested, that nucleoli in zygotes serve as a source of material that is gradually transformed into fully differentiated nucleoli. However, here these changes are interrupted by mitotic divisions. For this reason, nucleoli in fully grown oocytes and zygotes are called as "nucleolus precursor bodies – NPBs". Is the only function of oocyte (zygote) NPB to be a store of material for further formation of nucleoli in developing embryo? The invention of oocyte and zygote enucleolation method is helping us to answer this question. The enucleolation means that NPBs can be microsurgically removed from fully grown oocytes and one-cell stage embryos. The enucleolated oocytes mature up to metaphase II stage at the same frequency as controls. The karyotype of enucleolated oocytes is apparently normal. Interestingly, when MII enucleolated oocytes are fertilized or parthenogenetically activated, their pronuclei do not contain nucleoli and embryos typically cleave only to two-cell stage. When cytoplasts from enucleolated oocytes are used for SCNT, again pseudo-pronuclei are well visible but they do not contain nucleoli and embryos do not develop. This means that the somatic cell nucleolus cannot substitute for the original oocyte nucleolar material. Interestingly, when NPBs are transferred into previously enucleolated oocytes and these oocytes are then fertilized, their developmental competence is restored (live pups were obtained). On he other hand, when NPBs are transferred into zygotes obtained by IVF of previously enucleolated oocytes, only exceptionally these embryos reach the blastocyst stage. These

results indicate that NPBs are engaged in some processes that occur very soon after fertilization. The characterization of these processes is in progress.

Jean-Pierre Ozil *(INRA, France)*

The mammalian egg appears to transduce the duration, amplitude, and temporal presentation of the increase in the intracellular calcium concentration ($[Ca^{2+}]i$) upon fertilization. These Ca^{2+} parameters have important short-term effects on the initiation and completion of early events of egg activation, as well as much later consequences on the extent of development of peri-implantation and probably even on life.

While this developmental sensitivity of initial conditions is well acknowledged, it is still difficult to evaluate to what degree egg functioning is changed by small changes in the composition of the culture media. Egg activity is dynamic in nature and to understand how the formula of the culture media can impact the developmental outcome, a quantitative indicator that reflects differences in the time course of egg metabolism according to small changes in the formula of the culture media is needed.

Given the critical role of Ca^{2+} oscillations during fertilization, which persist for only a few hours and terminate with pronucleus formation, the characteristics of Ca^{2+} signal (frequency, number, amplitude and duration) offer a unique opportunity to detect changes in metabolic activity and to establish a functional linkage with the developmental outcomes.

For this purpose an algorithm was developed that makes it possible to decompose every single Ca^{2+} signal into two phases; firstly, the passive phase of Ca^{2+} release in the cytosol that is regulated by the open probability of the InsP3 channel according to the cytosolic InsP3 concentration and, secondly, the active phase of Ca^{2+} clearance that consumes ATP produced by the mitochondria. The ratio between the time duration of these two phases, for every single signal throughout the process of egg activation, provides a dimensionless profile of egg activity during the period of fertilization.

Such a ratio makes it possible to detect profound changes and differences in the activities of fertilized eggs incubated in either standard M16 or KSOM culture media that have the same components but with small differences in their concentrations.

Hence, the high sensibility of the self-sustained regime of Ca^{2+} oscillations to small changes in the concentrations of components between the two standard media M16 and KSOM offers new possibilities for linking changes in the Ca^{2+} regime at fertilization and the mechanism of genome programming, which has important biological and medical implications.

Pascale Debey *(INRA, France)*

Pascale Debey headed a research team from the Institut National de la Recherche Agronomique (INRA) devoted to studies of the nuclear dynamics and epigenetic changes in early mammalian embryos, particularly by fluorescence microscopy and computer assisted analysis. It is in that context that she developed a fruitful collaboration with Prof. Tarkowski and his colleagues.

From her initial experiments of labeling of chromatin by the vital dye Hoechst 3342, Pascale Debey pursued the study of the major reorganization of the embryonic genome that occurs during the whole preimplantation period, in correlation with epigenetic marks changes and developmental potential.

By microinjection of precursors her team was first able to determine the precise onset of Pol II- and Pol I-dependent transcription as well as the precise replication patterns in zygotes. She then analyzed on fixed and 3D-preserved embryos the distribution of centromeric and pericentromeric heterochromatin and the formation of chromocenters, which represent highly important elements of the nuclear organization, both from a structural and gene regulation point of view. This was performed i) first by indirect immunofluorescence detection of CENP (centromeric protein) and HP1β (heterochomatin protein 1), markers of centromeres and pericentromeres respectively, ii) then by fluorescent *in situ* hybridization (FISH). This required the setup of indirect immuno-fluorescence and FISH techniques preserving the 3D- organization of the nucleus. Confocal images capture was combined to computer assisted 3D reconstruction and quantitation of several parameters such as the number, volume and distribution of nuclear structures of interest.

These tools allowed to finely analyze the development-dependent nuclear distribution of asymmetric (parent dependent) and symmetric (parent independent) epigenetic marks such as DNA methylation, histones methylation and histones phosphorylation, over the same period.

Altogether these analyses allow defining several “transitions” along the developmental program, leading finally to blastocysts nuclei with characteristic features similar to those of somatic cells.

The same approach was then applied to embryos reconstructed by transfer of a somatic cell nucleus into an enucleated and activated oocyte (somatic clones). This allowed to i) highlight similarities and differences in genome organization as well as epigenetic marks distribution between clones and naturally fertilized embryos and ii) in several cases, establish a correlation between some characteristic structural or molecular features and the development potential of the clones.

More recently, thanks to the technical advances in microscopy, *ex vivo* observations of live embryos developed under the microscope stage could be made. Thus fluorescent signals could be followed directly during the transition from one stage to another, up to the blastocyst stage.

Janet Rossant *(University of Toronto, Canada)*

Janet Rossant began her talk by reminding the audience that she had collaborated in the 70s with Professor Tarkowski, when he was visiting Oxford from Poland and when she was still a graduate student with Richard Gardner. Together they showed that it was possible to generate haploid mouse blastocysts from bisected zygotes and published the results in a letter to Nature in 1976. Interestingly, this paper has recently received new citations as a forerunner of the current interest in derivation of haploid ES cells. In the rest of her talk, Dr Rossant described recent work from her lab and others that has helped define the signaling pathways and downstream transcriptional responses that lead to specification of the early cell lineages of the mammalian blastocyst, a subject that continues to intrigue Professor Tarkowski and the people trained in his lab. The formation of the blastocyst involves the segregation of extraembryonic (trophoblast and primitive endoderm) from the pluripotent epiblast cell lineage. All three blastocyst lineages can give rise to permanent stem cell lines *in vitro*, but only embryonic stem (ES) cells, derived from epiblast, retain the property of pluripotency.

It has been shown that the segregation of the inner cell mass from the trophectoderm is dependent on the activation of Hippo signaling in the inside cells of the developing morula, providing

a molecular explanation for the inside-outside hypothesis of blastocyst formation first postulated by Tarkowski decades ago. She presented data showing that the upstream Hippo signaling component, Merlin or Nf2, is required maternally to initiate Hippo signaling in the early embryo and that outer cell polarization blocks pluripotency upstream of Hippo signaling. FGF/ERK signaling levels are key to the gradual separation of pluripotent epiblast and primitive endoderm in the mouse blastocyst. Single cells from the early ICM are bipotent and capable of generating either ES or primitive endoderm-derived XEN cells in culture, dependent on FGF signaling response. She showed that this decision is not affected by altered Wnt signaling. Understanding the complexity of events occurring during establishment of pluripotency in the embryo can help elucidate the different states observed in pluripotent stem cell lines in vitro. Future experiments combining *in vivo* imaging with single cell gene expression analysis will provide ongoing insights into these important events in early development.

Berenika Płusa *(University of Manchester, UK)*

Three cell lineages emerge during mammalian preimplantation development. One of them – the epiblast – develops into the fetus. Two others lineages – the trophectoderm and the primitive endoderm – are the founders of extra-embryonic tissues that support and pattern the epiblast later in development. Interestingly, although lineage specification starts before blastocyst formation, our recent work as well as that of other groups have shown that a group of cells inside the blastocyst called the inner cell mass (ICM) cells retain the ability to contribute to all three lineages for at least 24 h after blastocyst formation. Complete loss of plasticity is observed only shortly before implantation. In other systems, cell differentiation and plasticity are considered to be antagonistic, since it is thought that cells gradually lose their plasticity during differentiation. However, the results from studying lineage specification during preimplantation development suggest that cells can retain plasticity for most of the time during lineage specification and that plasticity might be an important part of the differentiation process. Data obtained by Berenika Płusa group highlight the role of the early embryo microenvironment in lineage specification whilst retaining the capacity to sustain and induce plasticity in both embryonic and somatic cells.

Davor Solter *(Institute of Medical Biology, Singapore)*

Davor Solter described the importance of epigenetic mechanism in early development. Epigenetic alterations are increasingly recognized as causes of human cancers and disease. These aberrations are likely to arise during genomic reprogramming in preimplantation embryos, when their epigenomes are most vulnerable. Phenotypic variability in genetic disease is usually attributed to genetic background variation or environmental influence. However, Solter and his collaborators demonstrated that the deletion of a single gene, *Trim28*, from the maternal germline, on an otherwise identical genetic background, results in severe phenotypic and epigenetic variability that leads to embryonic lethality. He identified early, seemingly stochastic epigenetic variations in blastomeres of these embryos, suggesting misregulation of genomic imprinting in mice lacking maternal *Trim28*. By introducing a methodological advance, i.e. probing single cells for various DNA-methylation errors at multiple loci, they reveal failed maintenance of epigenetic marks results in chimeric mice displaying unpredictable, lethal phenotypes throughout development. Furthermore, they observed that pronuclear transfer can ameliorate such reprogramming defects, suggesting diagnostic and potential therapeutic applications. These results reveal the long-range effects of a maternal gene deletion on epigenetic memory and illustrate the delicate equilibrium of maternal and zygotic factors during nuclear reprogramming.

Małgorzata Kloc *(The Methodist Hospital, USA)*

Małgorzata Kloc described her work on the role of localized RNAs and their functions and the molecular factors involved in the determination of cell fates. Few years ago she also discovered a novel structural function of RNA in the organization and maintenance of the cellular cytoskeleton, which led to propose a novel hypothesis of binary function of mRNAs and the existence of binary phenotype of cells and organisms. Since the discovery of messenger RNA (mRNA) over half a century ago, the assumption has always been that the only function of mRNA is to make a protein. However, recent studies from her laboratory showed that mRNA plays a structural (protein-independent) role in maintaining the integrity of microfilament cytoskeleton. This implies that, at least in some cases, the cell or organism phenotype is in fact binary i.e. depends not only on the function of the protein but also on the autonomous function of its mRNA. Consequently, any loss-of-function method, which interferes with the integrity of mRNA will not only eliminate the function of a particular protein but also the function of the RNA itself. Thus, the conclusions about the function of the proteins, which are based on loss-of function technology may require re-thinking and re-evaluation. The fact that mRNA may have previously unforeseen structural functions, will change our view on traditional functions of RNA and will open new frontiers in the field of RNA studies and therapeutic development. Dr Kloc also developed novel light and electron microscopy techniques to study the cellular cytoskeleton and molecular components of the pathways structurally and functionally related to the cytoskeleton and RNAs. Her most recent work concentrates on the molecular pathways involved in regulation of cytoskeleton in immune response in organ transplantation.

Juan Aréchaga *(University of the Basque Country, Spain)*

The speaker addressed the mechanisms of cell migration during embryonic development and the process of tumor invasion, comparing the behavior of two pluripotent stem cell types: primitive germ cells (PGCs) vs. embryonal carcinoma cells (ECCs). Regulation of the former by factors related to embryonic microenvironments or the absence of such regulation in cancer progression might provide clues for understanding both mechanisms in a more holistic way, he proposed.

In mouse embryos, PGCs can first be detected at 7.5 days post-coitum (E7.5) as a small cluster of alkaline phosphatase positive cells in the extra-embryonic mesenchyme, close to the origin of the allantoids. From this region, PGCs move into the embryonic hindgut endoderm (E8.5) and, at E9.5 start to migrate actively from the hindgut wall and mesentery towards the growing urogenital ridges, where they finally colonize the developing gonads at E12.5. PGCs are motile at E10.5, whereas at E12.5 PGCs have lost their motile phenotype and begin to differentiate into gonocytes. Since matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) are known to be involved in cancer invasion and progression, he analyzed the expression of mRNA encoding nine MMPs and four

TIMPs in E10.5 and E12.5 PGCs by means of quantitative RT-PCR and immunocytochemistry, in order to clarify if putative changes in transcription levels might be associated with the migrating and the post-migrating phenotype of PGCs.

As a comparative pathological study, Prof. Aréchaga and colleagues have developed an experimental model to study teratocarcinoma invasion after injecting embryonic stem cells (ESCs) into adult seminiferous tubules of the mouse. In this part of the presentation, Dr. Aréchaga also showed distinct experimental approaches used in his laboratory to study the process of invasion of pluripotent stem cells (PSCs) *in vitro* and the emerging role of some cells such as peritubular myoid cells (PTCs), which express a number of factors that have been identified as pro-invasive signals in different cancer types. In this regard, he also focused his attention on the potential role of PTCs inside the reactive tumoral stroma, as precursors of cancer-associated myofibroblasts, in response to cell-derived signals like TGF-beta and PDGF. As consequence, the author emphasized that testis myofibroblasts could thus be a promising therapeutic target for testis germinal tumors.

Lynne Selwood *(University of Melbourne, Australia)*

Cleavage in all mammalian embryos results in the formation of an epithelium, in which some cells give rise to the embryo and others give rise to a nutritive lineage. In living mammals, the egg-laying Prototheria with meroblastic cleavage includes the monotremes, platypus and echidnas, and the Theria, with holoblastic cleavage includes the Metatheria, the marsupials, that retain the post ovulatory shell coat for most of gestation and the Eutheria, sometimes called placental mammals, in which only the pre-ovulatory egg coat, the zona pellucida, is retained during early cleavage. While these groups have not evolved directly from each other, but instead represent branches of extant mammals in the family tree, some features of early linage formation in these groups suggest how mammalian embryos evolved. Of particular interest are oocyte and zygote polarity, role of yolk and the mechanisms of extracellular matrix formation especially in monotremes and marsupials; the role of the zona pellucida and the embryonic egg coats; the timing and significance of appearance of cell-cell adhesion; the significance of the appearance of asynchronous cell division; the order of cell division in patterning the embryo; the possible roles of maternal determinants in monotremes and marsupials; characteristic features of the early lineages; and the variations in potency in the first lineages.

Andrzej K. Tarkowski *(University of Warsaw, Poland)*

Andrzej K. Tarkowski ['K.' stands for Krzysztof (Christopher, Kristoff), the name under which he is known to friends] reminded two studies that he had carried out in 1960/61 in Bangor where – as a postdoc - he had spent a year as a fellow of the Rockefeller Foundation. The two projects were devoted to: first – generation of mouse chimaeras by aggregation of two cleaving embryos and, second - to learning the fate of rat and mouse early embryos transferred reciprocally to the genital tract of the opposite species. While the first project ended successfully (birth of chimaeric pups), the second one did not – in both combinations the specifically 'foreign' embryos died at the time of implantation or soon afterwards.

In the next half a century the idea of combining these two experiments often came to his mind but other projects were more urgent and seemed to be more important. But finally this idea has materialized. In his presentation Tarkowski described the results of preliminary experiments aimed at generating mouse<->rat chimaeric embryos and at learning their fate after implantation. Surprisingly, inter-specific chimaeric embryos produced by simple aggregation of two cleaving embryos were able to survive beyond the middle of pregnancy despite severe morphological abnormalities and the large contribution of the alien cells to the trophoblastic 'shield' covering the foetus. More sophisticated methods were used to separate the chimaeric foetus from the mother by the trophectoderm specifically native to her. Other experiments are aimed at obtaining development of a non-chimaeric foetus in the mother belonging to another species. The results of these preliminary experiments are interesting in many respects and the project appears very promising.

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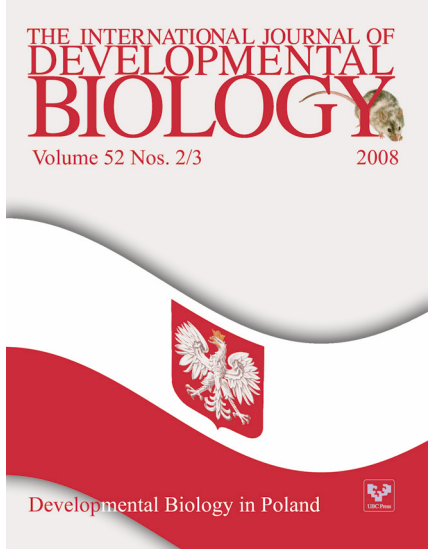

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