Dr Berenika Plusa
Lecturer
Michael Smith Building
Faculty of Biology, Medicine and Health
Oxford Road
MANCHESTER
M13 9PT
Tel: 0161 275 1563
Email: berenika.plusa@manchester.ac.uk

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Reviewer's report on the Habilitation Thesis of Dr Alexander William Bruce

Regulation of mammalian cell-fate: Insights from genomics-based investigation of transcriptional regulation and chromatin structure and studies of preimplantation mouse embryogenesis.

The work of Dr Bruce, summarized in his Habilitation Thesis, encompasses research done during two post-doctorate periods (one at the Wellcome Trust Sanger Institute and one at the Gurdon Institute, both Cambridge, UK) as well as the research performed in Dr Bruce's own research group that he established after moving to the University of South Bohemia in Ceske Budejovice (Czech Republic). The Thesis constitutes of two major research themes, both related to the overarching research interest, namely understanding how the cell fate is specified during mammalian development.

The original articles included in the first research theme: "Regulation of specific gene transcription and chromatin structure" prove that Dr Bruce made a successful transition from PhD student to more independent post-doctoral fellow. During this period of his career, Dr Bruce participated in several different projects, working as a part of the bigger consortium of researchers from different institutions, exemplifying his ability to performing as a team member in multinational endeavors. His work led to unexpected discovery that the REST/RE1 system is fundamental in modulating maturation and secretory function of neuroendocrine cells, in addition to the earlier known function of this factor as the inhibitor of neuron-specific gene expression outside the nervous system.

Dr Bruce demonstrated his abilities to transfer the techniques and knowledge absorbed during his post-doctoral fellowship in Sanger Institute into the new research field; the preimplantation mouse embryogenesis. In the second research theme of this Thesis: "Pluripotency and the acquisition of cell-fate in the preimplantation mouse embryo", the work of Dr Bruce aimed to understand how the first two cell fate decisions in mammalian embryos are controlled and executed. His work challenged the dogma existing in the field, that the first two cell fate decisions occur independently. Although the jury is still on whether this indeed is the case, his ideas invigorated the whole mammalian embryology community and led to completely new avenues of research, not only in Dr Bruce's lab but also in many others.

The work presented in the whole Thesis makes an original and significant contribution to the field of cell fate specification in mammalian development. Dr Bruce has proven that he is an independent, mature, versatile scientist, not afraid of any new challenges and that he has an excellent grasp of molecular intricacies of the cell fate control mechanisms in mammals. For the scientist at this stage of his career, his work is highly quoted (total 4372 citations to date; h = 14), highlighting the importance of his contribution to the field. Dr Bruce has been successful in securing the funding to carry on his work and has proven to be an excellent mentor for two outstanding PhD students that have recently defended their theses. Based on my overall assessment of the scientific and mentoring achievement of Dr Bruce, it is my pleasure to

recommend granting him the habilitation degree.

Question for the habilitation thesis defense:

1. Can the defendant provide the definition of pluripotency? Different parts of the thesis seem to use different classification of this term: sometimes in reference to the undifferentiated ICM, other times to the EPI.

2. If the role of maternal CDX2 is so important why does it seem not to be conserved between different mammalian species?

3. The data from Dr Bruce and Professor Zernicka-Goetz labs suggest that two cell fate decisions (TE vs. ICM and PrE vs. EPI) are not the separate events but hey remain interlinked. Other groups however, contested this claim. What are the possible reasons that other researchers failed to observe any link between two consecutive cell fate decisions in developing embryo?

4. On the same note, in the work published by the defendant's group (Mihajlović et al., 2015) the authors claim that when the cell ability to contribute to TE is blocked, the affected cells were less likely to contribute to PrE. This is slightly surprising, considering that none of their data show the statistically significant decrease in contribution of the affected cells to PrE, when the total number of cells within the embryo was considered. The effect of lowering rate of PrE cells was observed only when compared to the number of ICM cells. How can the defendant explain this discrepancy? Why there was no effect on PrE formation observed when total cell count was considered?

5. The author of the thesis declared that the majority of 4-cell stage embryos exhibit inter-cell heterogeneity in overall levels of a particular type of histone H3 methylation, namely R17/H3R26me. Is this really the case or is this phenomenon restricted to so called ME embryos (following the term from the original publication by Torres-Padilla et al., 2007) – a specific subset of 4-cell stage embryos?

6. Thus far, no one has demonstrated the presence of p-ERK prior the formation of "salt-and-pepper" distribution of EPI and PrE. Can one therefore claim that the FGF/ERK signalling is necessary for the initial recruitment of ICM cells to become PrE precursors? Is it possible that FGF/p38-Mapk signalling rather than FGF/ERK is crucial for the initiation of the process with further reinforcement from the latter?

7. Considering the results presented in the defender's work on Carm1 role in ES cells, which clearly demonstrated Carm1 occupancy on the Oct3/4 and Sox2 but not the Nanog promoter, is Nanog absolutely essential for ES cell self-renewal?

8. The applicant presented the convincing data upon how the forced down-regulation or up-regulation of Carm1 affected ES cells ability to self-renew and retain the undifferentiated state. In the applicant's opinion, is the Carm1 one of the gatekeepers, preventing the differentiation (via chromatin modification)? Is Carm1 down-regulated during differentiation of ES cells following standard protocols?

Berenika Plusa

UNIVERSITY OF WARSAW
FACULTY OF BIOLOGY
DEPARTMENT OF EMBRYOLOGY
PROFESSOR MAREK MALESZEWSKI, PH.D., D.SC.
HEAD OF DEPARTMENT OF EMBRYOLOGY

Warsaw, 22 November 2017

Reviewer's report on the habilitation thesis entitled „ Regulation of mammalian cell-fate: Insight from genomics based investigation of transcriptional regulation and chromatin structure and studies of preimplantation mouse embryogenesis" presented by Dr. Alexander Bruce

Dr. Alex Bruce graduated from the University of Leeds in 2004 where he received Ph.D. degree in biochemistry. Next he continued his scientific career as a research fellow at the Wellcome Trust Sanger Institute in Cambridge. After spending three years at the Chromatin Structure and Function Group, Dr. Bruce moved to The Gurdon Institute at the University of Cambridge, where he received a senior postdoctoral research associate position in the laboratory of Prof. Magdalena Żernicka-Goetz (Mammalian Development and Stem Cell Biology Group). After staying there for another three years Dr. Bruce moved to Czech Republic where he was offered the opportunity to establish his own research group at the University of South Bohemia in Českie Budějovice. Since then he is occupying the position of research group leader and lecturer. Dr. Alex Bruce is presenting a habilitation thesis entitled: **Regulation of mammalian cell-fate: Insight from genomics based investigation of transcriptional regulation and chromatin structure and studies of preimplantation mouse embryogenesis.**

Habilitation thesis submitted for review by Dr. Bruce comprises of 18 papers, which were published between 2006 and 2016 and an extensive (57 pages) introduction, which summarizes his scientific activity after his graduation. The thesis contains 12 original research papers, 3 review papers and two commentary articles. In two of the abovementioned research papers Dr. Bruce is the first author, in another two he is one of two or four equally participating authors, and in three papers Dr. Bruce is the last and corresponding author. One of these research papers is a report published in Nature ($IF5_{years}$ 43.8) by multi-institutional consortium. Other research papers coauthored by Dr. Bruce and incorporated into habilitation thesis were also published in very good or excellent journals, with IF_{5years} between 3.1 (*Reprod. Biomed. Online*) and 13.8 (*Genome Res.* – 3 papers). Review articles and commentaries included in the thesis were published in *Nature Rev. Genet.* ($IF5_{years}$ 42.4), *Curr. Opin. Genet & Dev.* ($IF5_{years}$ 5.9) and Reprod. Biomed. Online ($IF5_{years}$ 3.1 – 3 papers). Publications authored by Dr. Bruce were cited 3734 times excluding self-citations (WoS 04.17). His the most frequently cited paper (more than 2700 citations) is the report in Nature. In summary, the results of Dr. Alex Bruce's research were published in leading scientific journals and are have been frequently cited by other researchers.

Experimental papers, which comprise Dr. Bruce's habilitation thesis are grouped in two research topics, which reflect two stages of his scientific career. The first one: **Regulation of specific gene transcription and chromatin structure** is rooted in research which Dr. Bruce begun during his Ph.D. studies at the University of Leeds and continued after

ILJI MIECZNIKOWA str. 1, 02-096 WARSAW, POLAND
PHONE/FAX: (+48 22) 55-41-210
e-mail: maleszewski@biol.uw.edu.pl

graduation at Sanger Institute. The second topic: **Pluripotency and the acquisition of cell-fate in the preimplantation mouse embryo** covers the results of experiments which Dr. Bruce started at the Gurdon Institute and is continuing at the University of South Bohemia.

Dr. Alex Bruce Ph.D. thesis is related to bioinformatic identification of the binding sequences for neural transcription repressor protein REST in human and mouse genomes. REST binding motifs called RE1s are enriched at the sites of genome where the genes with neurosecretory functions are located. In his PhD. theses Dr Bruce demonstrated that REST is involved in the regulation of transcription of these genes. During the first stage of his postdoctoral career Dr. Bruce followed selected REST/RE1 system of the regulation of transcription as a model in genome-wide studies of the interactions between DNA and regulatory proteins in human cells. Application of the several advanced methods of the analysis of the DNA-protein interactions allowed Dr. Bruce to identify a large number of novel REST binding sites and target REST-regulated genes. In consequence he was able to demonstrate that REST has a role in the regulation of transcription of multiple genes, also outside the nervous system. Dr. Bruce also identified the existence of REST-genome binding affinities hierarchies. Additionally, he comprehensively analyzed the regulatory elements in the locus of the key human hematopoietic transcription factor SCL and managed to identify 6 novel regulatory elements.

While at the Sanger Institute Dr. Bruce was also involved in the studies undertaken by multi-laboratory consortium ENCODE, with targets the goal of functionally identifying all the regulatory DNA sequence elements in 1% of human genome. Within the frame of ENCODE project Dr. Bruce, together with a large number of other scientist from Sanger Institute, was able to correlate post-translation histone modifications with underlying DNA sequences and the function of these sequences in the regulation of transcription.

Therefore, during the early years of his postdoctoral scientific career Dr. Bruce carried successful studies on the regulation of transcription, making use of very modern experimental techniques. The results of his research enabled him to describe novel aspects of gene expression regulation by developmentally important transcription factors. He also managed to extend our knowledge on the role of post-translational histone modifications in transcription regulation, and he correlated certain patterns of histone modification with the genome architecture. Hence, Dr. Bruce's work at Sanger Institute was very fruitful and allowed him to participate in research, which significantly impacted our understanding of the regulation of the activity of genes.

The work on the role of the histone modifications in the control of the genetic activity turned Dr. Bruce's attention to the significance of this process for the determination of the cell fate during mammalian embryogenesis. In 2007 he moved to The Gurdon Institute at the University of Cambridge where he was offered a position of a senior researcher in the laboratory of Professor Magdalena Żernicka-Goetz, head of the one of the top groups studying the early mammalian development. At that time this research group demonstrated that in 4-cell mouse embryo blastomeres differ in global chromatin level of the post-translational histone H3 methylation at arginine 17 and 26, and that these difference correlate with various developmental fate of cells descending from respective blastomeres. In this context, the first research goal of Dr. Bruce was to analyze the significance of arginine-methyl- transferase Carm1 in the regulation of cell pluripotency. These studies were performed on mouse ES cells using biochemical and genomics methods, which could not be applied to mouse embryos due to inability to collect sufficient amount of embryological

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e-mail: maleszewski@biol.uw.edu.pl

material required for such studies. During this studies, Dr. Bruce and other researchers from Żernicka-Goetz group, demonstrated that very important pluripotency gens *Oct4*, Sox2 and *Nanog* in ES cells are (directly and indirectly) regulated by Carm1. This observation has a great significance for better understanding of mechanisms, which control cell pluripotency and differentiation by post-translational modification of histones.

Subsequently, when at The Gurdon Institute Dr. Bruce joined the ongoing research carried on by Żernicka-Goetz group, which was devoted to the study of the role of maternally derived Cdx2 mRNA during early cleavage of the mouse embryo. Zygotically-derived Cdx2 protein is known for its role in differentiation of the trophectoderm (TE) of the blastocyst. Żernicka-Goetz and collaborators proposed that maternal Cdx2 plays a role during the early preimplantation development preceding the formation of the blastocyst. They suggested that maternally derived Cdx2 is necessary for the proper apical-basal polarization of the outer cells in 16- and 32-cell stage embryos. Because the other research groups challenged this hypothesis, the goal of Dr. Bruce was to verify the presence of maternally provided Cdx2 mRNA and reexamine its putative function. The results of experiments confirmed the presence of maternal Cdx2 transcripts in early embryos and demonstrated that such transcripts are translated into Cdx2 protein. Development of embryos, which were depleted of Cdx2 mRNA by Cdx2-specific RNAi was arrested at 8- to 16-cell transition or at 16- to 32- cell transition and the blastomeres of these embryos underwent frequent apoptosis. These results strengthened the preposition that maternal Cdx2 mRNA has a role in ensuring proper establishment of apical-basolateral polarity of blastomeres that influences the specification of TE and inner cell mass (ICM) precursors from the 16-cell stage onward.

Because of the discrepancy between the results from different research groups, the role of maternal Cdx2 in development remained the subject of the heated debate. Henceforth the exchange of commentary correspondence took place between these researchers. Dr. Bruce wrote two commentary articles in which he discussed the possible reasons of apparently conflicting results and offered potential explanations for the observed discrepancies. He is very confident that his data and the conclusions on the role of maternal Cdx2 are valid. The important achievement of Dr. Bruce during his stay with Żernicka-Goetz group was also his coauthorship of three review papers on the mechanisms controlling cell differentiation during early mammalian development. One of these articles was published in Nature Reviews of Genetics – one of the most prestigious journals in the field.

Since 2010 Dr. Bruce has been carrying his research as an independent group leader at the Faculty of Science of the University of South Bohemia in Českie Budějovice. Establishing his research laboratory at this location was funded by several competitive research grants Dr. Bruce received from Czech and international funding institutions (two Marie Curie grants). Accordingly, Dr. Bruce demonstrated excellent abilities to attract financial support for his research. During this most recent period of his career Dr. Bruce's scientific interests have concentrated on the elucidation of the mechanisms, which in the mouse embryo govern the formation of the first two differentiated extraembryonic cell lines (trophectoderm – TE, derived from the outer cells of the embryo and primitive endoderm – PE, which forms from the inner cells - ICM) and a pluripotent group of cells, which during further development build the body of the fetus and the most of fetal membranes (inner cells derived epiblast – EPI). His first experimental hypothesis was that three abovementioned cell lineages are formed not in two functionally independent steps, but in

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e-mail: maleszewski@biol.uw.edu.pl

one interlinked sub processes. Thus, he made an effort to clarify whether differences between the inner blastomeres of the embryo, which ultimately lead to the formation PE and EPI of the blastocyst may be related to their origin from two rounds of differentiative divisions during which inner cells of the blastocyst are separated from the outer TE precursors. In a series of diligently planned and meticulously performed experiments Dr. Bruce managed to demonstrate that interference with the first stage of cell differentiation in the embryo (TE vs. ICM) influences the second differentiation process (EPI vs. PE). Consequently, he proposed that positional clues, which inhibit Hippo signaling in outer cells of the embryo and thus induce these cells to differentiate into TE, concomitantly prime the inner cells, which arise from differentiative divisions at the 8- and 16-cell stages, to differentiate into PE. So heterogeneity of the ICM cells may result from the length of the exposition of parental blastomeres of the inner cells to such clues, since TE-priming of inner cells derived from the 5th cleavage division (second differentiative division of outer blastomeres of 16-cell embryos) is longer than inner cells created during 4th division. Thus, Dr. Bruce provided evidence that two cell-fate decisions, which occur in the preimplantation embryo, are indeed interlinked. On the basis of these results Dr. Bruce proposed an integrated ("time-inside time-outside") cell-fate decision model explaining the formation of EPI and PE lines in mouse blastocyst.

Another aspect of Dr. Bruce studies involves the role of Rho-associated protein kinases (Rock1/2) in early mammalian embryogenesis. These kinases are known for their role in the regulation of embryo compaction, however their exact function in this process was not known. Previous reports on the effects of Rock1/2 inhibition on the preimplantation development presented conflicting results. Dr. Bruce decided to examine the Rock1/2 inhibited embryos, and found that they failed to form a blastocyst due to inability of outer cells to specify the TE lineage. He discovered that defective blastocyst formation was a consequence of the breakdown of apical-basal polarity in outer cells, which were unable to form a functional epithelium. Dr. Bruce found that result of Rock1/2 inhibition was mediated by miss-localization of the hippo-signaling pathway activator Angiomiotin (Amot) and in consequence ectopic activation of the hippo pathway in outer cells of the embryo, what prevented the expression of TE specific genes in these cells. Accordingly, Dr. Bruce demonstrated that Rock1/2 is directly involved in the regulation of the developmental fate of inner and outer cells in preimplantation mouse embryo by controlling formation of apical-basal polarity in outer cells.

Most recently, Dr. Bruce has studied the mechanisms underlying the second cell fate decision in the embryo – the formation of the PE within ICM of the blastocyst. It is known that ICM lineage specification is under the control of Fgf/MAPK pathway in which the extracellular signal regulated kinases 1/2 (Erk1/2) plays an important role. On the basis of the results from ES cell studies Dr. Bruce hypothesized that the related mitogen activated kinases (p38-Mapks) may be also involved in the regulation of PE formation in the blastocyst. He observed that inhibition of p38-Mapk activity in embryos effects in significantly reduced number of PE cells. These data indicated a role of p38-Mapk in PE differentiation. Dr. Bruce managed to demonstrate that p38-Mapk activity is required during early blastocyst development, and that its role is distinct from previously known kinases involved in blastocyst maturation. He also has shown that p38-Mapk acts downstream of Fgf/Fgfr signaling. Taken together these results demonstrated novel regulatory role of p38-Mapks in regulation of the fate of cells in the blastocyst. On the basis of his results Dr. Bruce proposed

ILJI MIECZNIKOWA str. 1, 02-096 WARSAW, POLAND
PHONE/FAX: (+48 22) 55-41-210
e-mail: maleszewski@biol.uw.edu.pl

an original model of the molecular mechanisms, which control the cell fate within ICM of the mouse blastocyst.

In conclusion there is no doubt that the research included in Dr. Bruce's in habilitation thesis very significantly extends our knowledge on the mechanisms regulating the cell fate during mammalian development. Publications incorporated into the body of his habilitation demonstrate that Dr. Bruce played a major role in the progress of his research discipline. These publications are also a proof of Dr. Bruce scientific excellence, his scientific maturity and the ability to carry on his studies as a fully independent scientist.

Accordingly, taking into account that I have very high opinion about the quality of Dr. Bruce habilitation thesis, I certify that he fully deserves the degree of the habilitated doctor.

Prof. Dr. hab. Marek Maleszewski

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ILJI MIECZNIKOWA str. 1, 02-096 WARSAW, POLAND
PHONE/FAX: (+48 22) 55-41-210
e-mail: maleszewski@biol.uw.edu.pl

Report on the Habilitation thesis of Alexander William Bruce
by
Professor Martin H Johnson FRCOG, FRSB, FMedSci, FRS

In this report, I focus on second theme of Alex Bruce's thesis, since it is this theme with which I am most familiar and it is also the theme that relates to his current work.

Alex has made several substantial contributions to the subject of pluripotency and the acquisition of cell-fate in the preimplantation mouse embryo. His most substantial contribution has been the evidence that he has produced that favours a lineage-dependent mechanism underlying the second major decision during development, namely the origin of epiblast and hypoblast (I use here the more acceptable terminology than primitive endoderm used by Alex: see Johnson and Selwood, 1996 The nomenclature of early development in mammals. Reprod. Fertil. Devel. 8, 759-764.; Question 1 – why are you using this traditional but less rational terminology?). Thus, the results from two experiments support a role for the sequence of cell allocation to the inside being important i.e. the integrated cell-fate model: see sections 3.8 and 3.10 (Question 2 – the critical role of the FGF receptor for the formation of the hypoblast cells comes out clearly in these experiments; why is it not described how the expression difference of this receptor in epiblast and hypoblast cells is regulated, especially temporally, but also mechanistically?).

The second area in which Alex has made a substantial contribution has been in the analysis of the development of cell polarity of 8-cell blastomeres and the role that this process plays in generating cell diversity. Thus, the roles that rho-associated kinases 1 and 2 and the localisation of amot play in the establishment of polarity (section 3.9) adds a detail to our knowledge about how the Hippo-signalling pathway is suppressed during polarisation (Question 3 – how exactly is amot directed, and then linked, to the pole by the kinases during the normal polarisation process?).

In a second series of experiments, Alex has examined the molecular basis of the initial setting-up of polarity in 8-cell blastomeres. In the first of these experiments (section 3.5), Alex examined the role of carm 1 in influencing 4-cell fates in the embryo, and describes how in embryonic stem cells carm 1 knock down results in the loss of pluripotency and the reduced expression of both Oct4, and sox2 directly, and nanog indirectly; the reverse outcome applies if overexpression of active carm 1 was achieved. These experiments offered an explanation as to how the heterogeneous expression of carm 1, and the associated changes to histone methylation, might influence cell fate in the 4-cell blastomeres (Question 4 – how exactly does this influence the cytoplasmic allocation mechanisms during polarization, given that all cells during cleavage normally express oct 4 and the appearance of nanog is a relatively late event in the process of cell diversification?). In a second, experiment, Alex examined how the maternally inherited Cdx2 mRNA/protein might influence polarization (section 3.6), and concluded that the inheritance of differing amounts of cdx2 mRNA/protein might push a cell towards (higher levels) or away from (reduced

levels) a trophoblast cell fate, in the latter case by interfering with the process of polarisation (Question 5 – how exactly since the expression of cdx2 is a relatively late event in the current model of the sequence of polarisation?).

Overall, I can say with confidence that on the basis of his published work, Alex has amply fulfilled the requirements for Habilitation.

[redacted]

Martin Johnson