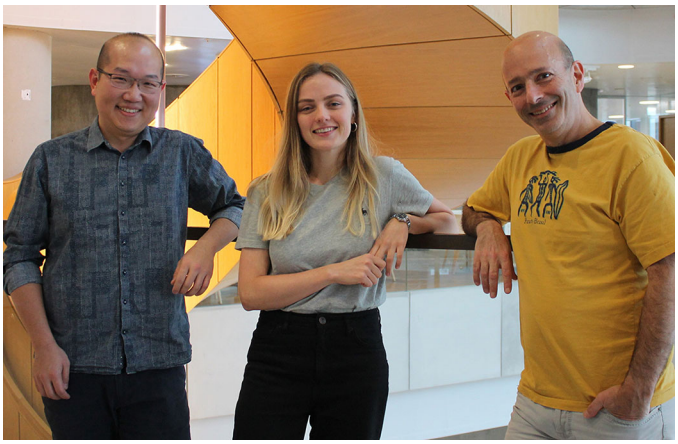
© 2019. Published by The Company of Biologists Ltd | Development (2019) 146, dev186635. doi:10.1242/dev.186635

INTERVIEW

The people behind the papers – Madeleine Linneberg-Agerholm, Yan Fung Wong and Josh Brickman

Our understanding of lineage decisions in early human development has been greatly aided by embryonic stem cell lines, which avoid many of the practical and ethical difficulties of *in vivo* material. A new paper in Development exploits naïve human embryonic stem cells to generate *in vitro* models for the extra-embryonic endoderm. We caught up with first authors Madeleine Linneberg-Agerholm and Yan Fung Wong, and their supervisor Josh Brickman, Professor of Stem Cell and Developmental Biology at the Novo Nordisk Foundation Center for Stem Cell Biology (DanStem) in Copenhagen, to hear more about the work.



Yan Fung, Madeleine and Josh (L-R)

Josh, can you give us your scientific biography and the questions your lab is trying to answer?

JB Since the beginning of my PhD, I have been focused on the transcriptional basis for cell identity. Following a brief foray into the music industry as both a DJ and journalist, I began a PhD under the guidance of Mark Ptashne at Harvard University, where I worked on general mechanisms of transcriptional synergy and cooperativity. By the end of my PhD, I felt the need to take this work into a more biological context. To this end, I trained as a post-doctoral fellow with Rosa Beddington at the National Institute for Medical Research in London, where I began to work with a combination of early embryos (mouse and frog), and embryonic stem cells (ESCs), to explore the means by which transcription controls anterior specification. In 2001, I started my own group at the Institute for Stem Cell Research (now the MRC Centre for Regenerative Medicine), University of Edinburgh, where I used a combination of ESC models and early embryos to deconstruct the transcriptional basis for lineage specification and potency, focusing on endoderm induction and patterning. In those early years, we used *Xenopus* embryos with parallel experiments in ESCs as a rapid means to understand conserved mechanisms regulating both pluripotency and differentiation. However, with time, my lab has unfortunately lost touch with its amphibian roots as the lure of stem cells became too much for my students to resist.

One of the most important observations we made in those early years was that ESCs could be used as means to model early development in the primitive endoderm and to trap spontaneously arising transcriptional states in which cells were reversibly and functionally primed for differentiation. This led us to the notion that self-renewing cell culture models could be used to trap intermediate, or uncommitted, transcriptional states in differentiation. We see these states as analogous to transition states for lineage specification, and we have used these models to identify mechanisms governing these reversible transcriptional changes. At the same time, we also began to view karyotypically normal, embryo-derived cell culture as a means to trap decision points in differentiation with a capacity for proliferation. We exploited this idea as a way to isolate and expand lineage-restricted progenitors from differentiating ESCs in both the definitive and later primitive endoderm lineages.

In 2011, my lab relocated to the DanStem at the University of Copenhagen, where we continue to focus on the transcriptional basis for cell fate choice. In particular, we're interested in the basic mechanisms regulating transcriptional heterogeneities in early embryos and differentiation, how gene regulatory networks can be used to explain the differentiation competence and self-renewal of stem and progenitor cells, and how transient transcriptional states become committed in differentiation. Of course, a number of these questions concern the interface of gene regulatory networks with signalling and this has been a major focus of our recent work, including this new paper.

Before concluding, I think I should tell a short story about the origin of this work. About ten years ago, we had some translational funding to apply our work on mouse endoderm differentiation to human ESCs. I used this money to support a student (Maurice Canham) who was finishing his work in the lab on mouse primitive endoderm priming and wanted to take on this translational project. At the time, all the available human cells were primed pluripotent cells. While he was adapting our culture conditions to human ESCs, he decided to dump human endoderm differentiation media on mouse ESCs and see what happened. He observed this remarkably homogenous differentiation to a cell type he thought resembled a slice of pizza and, therefore, referred to them as pizza cells. At the time we were convinced that 'pizza cells' were probably primitive endoderm, but it took another PhD student (Kathryn Anderson) years to prove this was the case, to test the activity of these cytokines side by side on primed and naïve cells, and to work out the conditions for the passaging of naïve extra-embryonic endoderm (nEnd). Years later, naïve human pluripotent cells became available, and we were finally able take this work back to the human cells that it started with.

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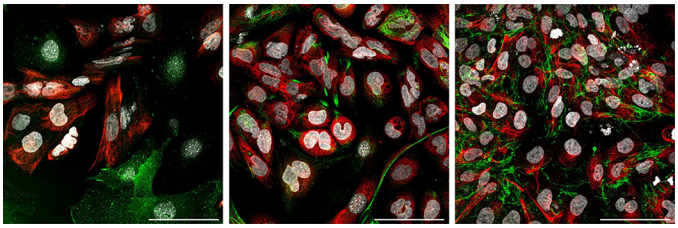

Immunofluorescence of human nEnd stained for endoderm and basement membrane markers. Left: E-cadherin (green), vimentin (red), GATA6 (white). Middle: AFP (green), collagen IV (red), GATA6 (white). Right: fibronectin (green), vimentin (red), GATA6 (white).

Madeleine and Yan Fung, how did you come to work with Josh and what drives your research today?

ML-A Although originally from Denmark, I did my undergraduate degree in the UK. I became really interested in early mammalian development as a result of my bachelor's thesis project in Ryohei Sekido's group at the University of Aberdeen, working on Y-linked sex-specific epigenetic modifications in mouse ESCs. After four years abroad, I got homesick, but luckily found Josh's group in Copenhagen, and was able to return to begin a master's degree under his supervision. In Josh's group, I was trained by Fung who became my day-to-day supervisor and taught me hESC culture. I was quite fortunate to join when I did, as it was an exciting time both for the group, as they were about to publish the story of the context dependence in mouse endoderm differentiation (Anderson et al., 2017), and also in the field, as a number of human naïve ESC papers had recently come out. I think what drives my research today is trying to fill in the most fundamental steps in human development and reconcile what we know in other species with ourselves.

YFW I finished my PhD in Hong Kong where I studied gene regulatory networks and organ patterning using *C. elegans* as a model organism. I then applied to work with Shinichi Nishikawa at the RIKEN Center for Developmental Biology, where I used human cell lines and primary cells as disease models to study epigenetic regulation. During that time, I had the chance to meet stem cell biologists from all over the world, including a former PhD student of Josh's, Kathryn Anderson (one of the authors in this paper), and she convinced me to think about going to his lab. I then applied to Josh's lab and met with him in Washington DC, after which he encouraged me to visit the lab in Denmark. In 2013, shortly after the group had relocated from Edinburgh to Copenhagen, I came to visit the new centre, DanStem. Attracted by the passionate people in the group, the newly established research institution, and Josh's impressive work using stem cell culture systems as models to understand the transcriptional basis for lineage choice, I joined the lab.

What makes endoderm induction in the mouse context dependent, and before your study what was known about its conservation in humans?

JB, ML-A & YFW We believe that the context dependence we originally saw in mouse was determined by changes in the enhancer landscape between naïve and primed pluripotency. The interaction of Wnt and Nodal-related TGFβ signalling with the set of enhancers primed in these cell types would determine the trajectory of differentiation. In our mouse nEnd paper (Anderson et al., 2017), we found that there was a correlation between enhancer accessibility and definitive endoderm versus primitive endoderm lineage differentiation. This is a remarkably similar idea to our recent thoughts on specificity of FGF/ERK signalling (Hamilton et al., 2019). Here, we found that ERK directly regulates enhancers, but that the activity of ERK on enhancers is likely to depend on pre-bound transcription factors, that don't in themselves activate transcription, but prepare the available differentiation trajectories a cell can take when exposed to a signal. We believe a similar mechanism must be at work here with respect to endoderm enhancers that respond to Nodal/Wnt signalling in either naïve or primed pluripotency, with these pathways acting on different pre-wired transcriptional circuits that are stimulated by the same signalling pathways, but in different pluripotent states.

At the time we started, it was known that it was possible to culture human naïve cells and that their culture was usually dependent on FGF/ERK inhibition. However, the role of FGF/ERK described extensively in mouse primitive endoderm and epiblast segregation appeared not to be conserved in human embryos. As we believe that inhibiting primitive endoderm differentiation was a primary function of ERK in naïve ESC culture, we wondered how one could reconcile these observations.

Can you give us the key results of the paper in a paragraph?

JB, ML-A & YFW We found that the context dependence we observed in mouse, in which activation of Wnt/Nodal and LIF signalling could promote lineage-specific endoderm differentiation (i.e. primitive versus definitive) based on the developmentally proximal state of the starting culture, was conserved in human. Thus, human naïve pluripotent cells, which resemble the pre-implantation embryo, differentiated to primitive endoderm in response to these pathways, whereas primed pluripotent cells, which resemble the pre-gastrulation-stage epiblast, gave rise to definitive endoderm. We were then able to use this primitive endoderm differentiation model to show that the role of FGF/ERK in specifying this early lineage, at least *in vitro*, was conserved. Importantly, we were able to establish conditions for the expansion of these *in vitro*-derived cells to establish a culture/stem cell system for human hypoblast (as the human primitive endoderm is known). As trophoblast stem cells have recently been produced in human and naïve ESCs are thought to represent epiblast, this new culture system means that there are now human cell lines/*in vitro* models for all three lineages of the blastocyst.

What changes between the naïve and primed states to direct what kind of endoderm ESCs can give rise to?

JB, ML-A & YFW This was discussed above with respect to mouse. We believe it is the gene regulatory network in these different states that provides that platform on which the signalling pathway acts. The transcription factors expressed in these different stages of pluripotency could be sitting on distinct enhancers preparing cells to adopt different trajectories of differentiation in response to the same signal. It's as if the transcription factors are laying down a road along which the cells can progress in differentiation in response to these signals. When cells transition from naïve to primed pluripotency the road is diverted and signalling pushes cells in this new direction.

What pressing questions do you think your nEnd cells will be particularly suitable for addressing?

JB, ML-A & YFW We think these cells will be particularly useful for the study of human primitive endoderm patterning and differentiation. They will be an excellent tool for studying how regulatory networks become stabilised in self-renewal in the endoderm and how these can then initiate patterning. As nEnd

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represents the third cell type from the blastocyst, they will also be very useful in experiments designed to determine the self-organising properties of early embryonic cells in order to generate embryoids. Finally, they provide a system in which to study the differentiation of the primitive endoderm and understand how it compares to the definitive endoderm.

When doing the research, did you have any particular result or eureka moment that has stuck with you?

ML-A For me, there were three moments that really stood out for different reasons. The first one was when I saw the first naïve colony after chemical resetting from primed hESCs. It was the first 'big' experiment that I did, both on this project and also in my time in the group, so that was a big moment of success for me. The second was when I saw the first patch of primitive endoderm after my first ever differentiation from naïve hESCs. That was when we knew the project was going somewhere, and it was likely that endoderm specification between mouse and human was conserved. The third was when we figured out the expansion conditions for primitive endoderm to make nEnd, which I was stuck on for easily half a year. When the expansion worked, I started to see the future potential of what I was doing beyond this paper and all the exciting experiments that it could lead to.

YFW Expansion, and the excitement of getting expansion working!

When the expansion worked, I started to see the future potential of what I was doing beyond this paper

And what about the flipside: any moments of frustration or despair?

ML-A A lot! It was really challenging having to learn the most basic aspects of doing research at same time as having such an ambitious project, from cloning and doing my first RT-qPCR to learning R (thank you, Stack Overflow). But I think that just made it all the more rewarding, or at least that's how I feel now.

YFW When I found out that I could not detect HHEX expression in differentiating primitive endoderm from human naïve ESCs, I thought we had a problem with the cells. However, based on the single cell transcriptome data on the human blastocyst it turned out to be true.

So what next for you two after this paper?

ML-A I graduated with my master's degree this summer and now I'm taking a year 'off' working as a research assistant in the lab. My plan is to start my PhD with Josh next year. I am continuing with human nEnd projects, but I've started working with mouse endoderm as well, as it offers a whole new world of experimental possibilities.

YFW Besides this work, I am finishing other projects related to foregut endoderm expansion and differentiation to visceral organs, including pancreas and liver. The main focus is to understand how extrinsic signals influence transcriptional networks or chromatin accessibility. I am interested in how these networks impact the choice these progenitor cells make between self-renewal and lineage specification. I hope this work will bring us one step closer to understanding human embryonic development and perhaps translating this knowledge into strategies for regenerative medicine.

Where will this work take the Brickman lab?

JB As a lab we are very excited about these cell lines. We are excited by the potential of exploiting nEnd to explore the self-organising properties of human primitive endoderm, both on its own and when recombined with other cell types. We are also excited about using nEnd as a model to understand human visceral endoderm patterning.

Since I first started my lab, I have worked on gastrulation-stage endoderm patterning and using ESC differentiation as a model for this. While we have just begun this sort of work in human models, nEnd will complement them nicely. We are looking forward to using these cells to explore 'extra-embryonic' endoderm differentiation in human.

Finally, the *in vitro* model we describe here for human primitive endoderm differentiation will provide us with an excellent platform to collect evidence for our ideas about signalling context. How does the enhancer state or gene regulatory network in naïve and primed pluripotency determine signalling response?

Finally, let's move outside the lab – what do you like to do in your spare time in Copenhagen?

ML-A Just like in the UK, it definitely depends on the weather. If it's nice, I like going for walks with my dog in a forest north of Copenhagen called Dyrehaven, which is actually a UNESCO World Heritage Site. On rainy days, I like to try and find the best ramen place in Copenhagen (currently Ramen To Bíiru) or stay at home watching '90s rom-coms and playing video games.

YFW Hygge with family and friends, discussing the big and small things in life.

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