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On the shoulders of Hubrecht: From embryos to stem cells

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A B S T R A C T

One hundred years of the Hubrecht Institute were celebrated in May 2016 with the organization of a one-day symposium “From embryos to stem cells” on the Uithof Campus, Utrecht, the Netherlands. Nine distinguished speakers were invited. They all represent a research branch originating from the passion of Institute founder, Ambrosius Hubrecht, for embryology:, regulation of gene expression, genome structure and function, embryonic and adult stem cells, nuclear reprogramming, and understanding cancer and other diseases using model organisms.

The centennial symposium not only retraced the history of the Institute and of modern developmental biology, but was also a tribute to basic research. From there, avenues to therapeutics are being developed and implemented. The symposium was organized, introduced and chaired by Jeroen den Hertog and Alexander van Oudenaarden, the present Directors of the Institute, who also stand on Hubrecht's shoulders.

1. Introduction

1.1. Hubrecht and descriptive embryology

Born in 1853, a scholar from a wealthy Utrecht family, Ambrosius Hubrecht spent most of his adult life studying, describing and reporting on the embryonic development of many placental mammals, including hedgehogs (that were for a long time considered to be marsupials) (Fig. 1). He financed and undertook a number of expeditions to gather new animals and embryos, very much in the footsteps of Darwin whom he admired. He focused on the importance of comparative embryology to reveal evolutionary relationships between species. This evolutionary view of the animal kingdom was brave as it opposed the Christian belief dominating Dutch society at that time.

Hubrecht's lab was set in the basement of his house at Janskerkof in the center of Utrecht. In 1911, he founded the “Institut International d′Embryologie” (now the ISDB) as a repository for his embryological collection and a communication network for embryologists world wide. He died in 1915.

Confronted with the potential danger of losing this precious collection and associated scientific knowledge, the Royal Netherlands Academy of Arts and Sciences (KNAW) made the Hubrecht Laboratory an official KNAW institute in 1916, with the mission to primarily make materials and expertise available to the growing international community of developmental biologists.

1.2. Nieuwkoop and experimental embryology

The 1920s witnessed the advent of experimental embryology exemplified by Spemann's and Mangold's transplantation experiments (Spemann and Mangold, 1924) for which Spemann was awarded the Nobel Prize in 1935 (Spemann, 1965). With the arrival of Christiaan Raven and his research student Pieter Nieuwkoop in 1947, the mission of the Hubrecht Laboratory changed from descriptive to experimental embryology. In particular, Pieter Nieuwkoop undertook his pioneer investigations on embryonic inductions for which he is considered a giant of 20th century embryology (Nieuwkoop, 1969a, 1969b; Nieuwkoop et al., 1952).

Pieter Nieuwkoop became director of the Hubrecht Laboratory in 1953. He pursued a mission of making the Hubrecht Laboratory a suitable place for experimental studies in developmental biology. He convinced the KNAW that the Hubrecht Embryological Collection was

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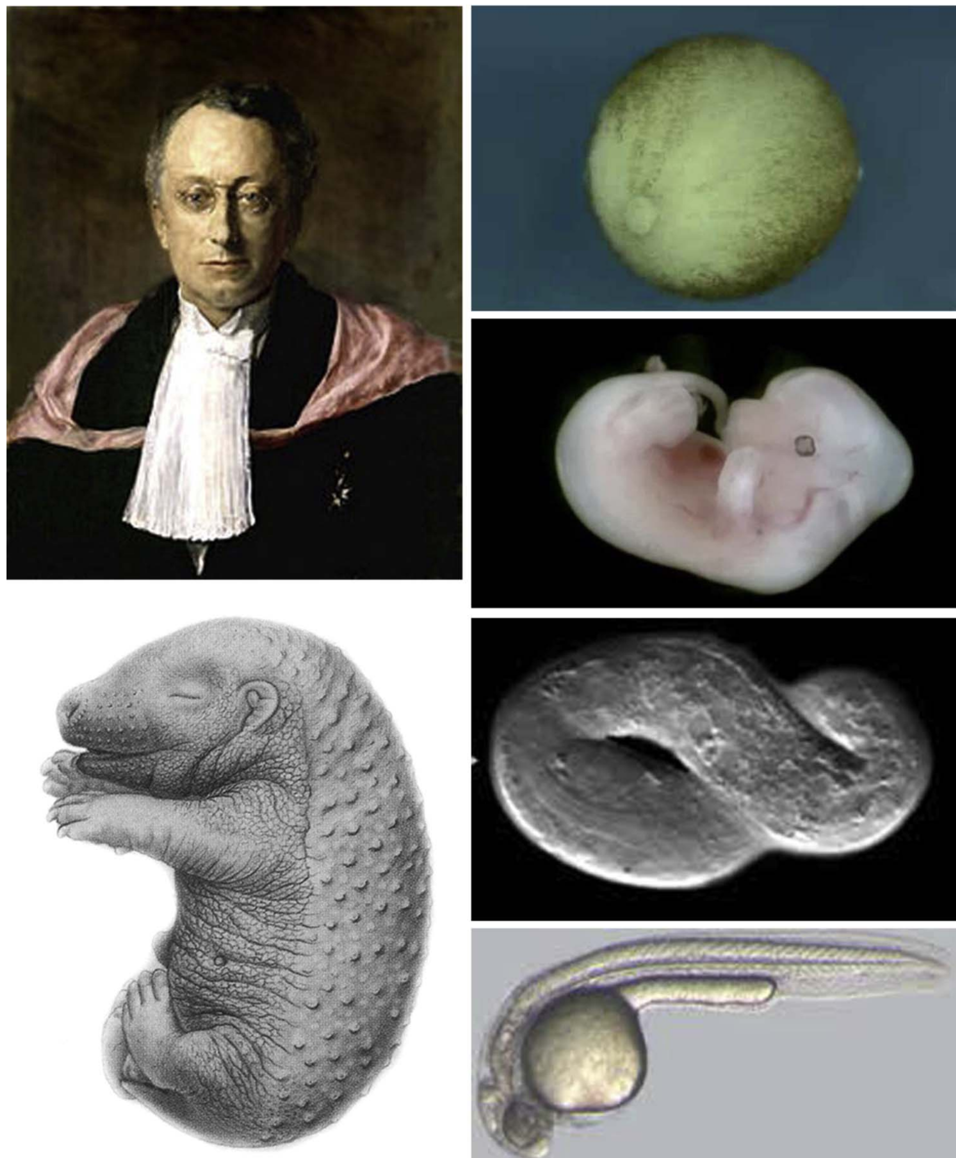

Fig. 1. Embryology at the Hubrecht laboratory. Left: Portrait of Ambrosius Hubrecht and pencil drawing of a hedgehog embryo (J. J. Prijs, 1910. Hubrecht archives, Utrecht University museum). Right (top to bottom): Photographs of Xenopus, mouse, C.elegans and Zebrafish embryos.

a precious collective resource world-wide and that the network of embryologists centered at the Hubrecht Laboratory was key to future research. He not only obtained the KNAW approval for a new building, but also ensured financial support for the appointment of additional principal investigators. The first settlement in the country fields of what was to become Utrecht University campus, de Uithof, became a reality in the early 1960s

1.3. De Laat: the turbulent years

In the 70ies, the choice of priorities in the research program was heavily debated in the Hubrecht Laboratory. After years of turbulence, Pieter Nieuwkoop resigned and Siegfried de Laat became director in 1983. The lab became a multi-disciplinary research institute with new research lines and methodologies, including the use of embryonic stem cells, molecular biology and gene expression regulation, in the hands of new research teams.

1.4. Plasterk and Clevers: The genome and adult stem cells

In the second half of the 90ies, plans were made for the construction of a new, larger building at the back of the Hubrecht Laboratory. This new building was inaugurated when Ronald Plasterk became director in 2000. With him, the lab entered the genomics era exemplified by RNAi and genome-wide screens in C.elegans and Zebrafish (Fig. 1). Hans Clevers was appointed as second director in 2003, bringing with him research on adult stem cells. The Hubrecht Laboratory became the Hubrecht Institute.

1.5. van Oudenaarden: Onward

When Hans Clevers was nominated president of the KNAW in 2012, Alexander van Oudenaarden became the director of the Hubrecht Institute. With him came quantitative and single cell biology, thus strengthening the ever-growing expertise of the Institute. In order to increase the research capacity further, plans for the construction of an additional building were made.

This new building was opened in the summer of 2015, just in time for the 100 years anniversary of the Hubrecht Laboratory/Institute. The celebration of this anniversary started with the visit of His Majesty King Willem Alexander who unveiled the sculpture made by Jeroen Korving and dedicated to this Centennial. This was followed by the one

day-symposium “From embryos to stem cells”. 500 participants (many locals but some from abroad) gathered to listen to a panel of nine excellent speakers celebrating 100 years of research and innovation in Developmental Biology and beyond, all standing one way or another on the shoulders of Ambrosius Hubrecht.

Here, we report here on the presentations that were given during the centenial symposium on May 19, 2016.This will be part of a larger issue of Developmental Biology dedicated to the research at the Hubrecht Institute, the second of the kind (1999, 43:583-775).

2. From experimental embryology onward

2.1. From experimental embryology to nuclear reprogramming and cell replacement prospects. ***Sir John Gurdon*** *(Cambridge University, UK)*

It was very fortunate and appropriate to have John Gurdon as a first speaker of this Centennial symposium. John Gurdon embodies the progress made in understanding reprogramming of the genome, starting from the wish to understand the process of development of Xenopus early embryos, and paving the way to mastering the ability to reprogram a somatic cell. This understanding contributed to the advent of the induction of pluripotency in somatic cells by only a few transcription factors, an achievement rewarded by the Nobel Prize in 2012 that he shares with Shinya Yamanaka.

John Gurdon started his presentation by reminding the audience of the groundbreaking contribution of Pieter Nieuwkoop's research on amphibian embryos. Amphibians were a much studied system at that time. It is the remarkable ability of the Xenopus fertilized eggs to develop into a whole organism without their mother's contribution that incited John Gurdon to investigate the fundamental mechanisms of gene control during development.

This had been started by Briggs and King in 1952 (Briggs and King, 1952), who showed that normal hatched tadpoles can be obtained after transplanting the nucleus of a blastula cell to an enucleated egg of *Rana pipiens. John Gurdon capitalized on this finding. He performed nuclear transfers into Xenopus eggs, and discovered that* nuclei from intestinal epithelium of Xenopus feeding tadpoles could be reprogrammed to support the development of more complete embryos with identifiable tissues when their nuclei were re-transplanted into enucleated eggs.

Unfortunately, most of the resulting blastulae looked only partially normal and died after 24 h, even if normal development to term was sometimes observed. Interestingly, cells from partial blastulae that survived after one day could be reprogrammed to participate in several host tissues after grafting. That suggested that these cells could be reprogrammed and grow and differentiate in spite of their deficient initial reprogramming.

The fact that the majority of somatic nuclei failed to reprogram after transplantation to eggs was attributed to damage to the somatic nuclei during the procedure, and to sub-optimal composition of the recipient cytoplasm. An improvement was to transplant the nuclei to the germinal vesicle of an oocyte, preferably a first meiotic oocyte. Reprogramming by oocytes requires a hierarchical sequence of events and the molecules required are at high concentration in the oocyte germinal vesicle (Gurdon, 1986).

Regarding the somatic nuclei transplanted into oocytes, they switch their pattern of gene expression to repress differentiation genes and to adopt the oocyte transcription program. They undergo a series of successive activation steps, ending up with deposition of active histone marks and novel transcription (Jullien et al., 2011). The efficiency of reprogramming is therefore limited by both the resistance to reprogramming and the stability of cell differentiation. These events are contributed to by DNA methylation, epigenetic memory, and transcription factor residence and exchange. The residence time of transcription factors on the DNA (versus their exchange) as well as their concentration are fundamental determinants of cell fate and early embryonic development. In this regard, the mitotic phase was shown to be favorable to reprogramming because the chances of transcription factor exchange are higher. As such, it constitutes a window of opportunity to modulate gene expression, explaining the mitotic advantage for reprogramming. Altogether, loosening the nucleosome composition and a better access of transcription factors to DNA lead to a decrease in resistance to reprogramming and an increase in stability after reprogramming. This, in fact, obeys the general principle of development where an embryonic cell always divides before entering a new path of cell fate.

John Gurdon ended his presentation by expanding on a therapeutic application of the understanding of reprogramming that promises to cure a particular form of age-related macular degeneration. This illustrates the progress made since the original questions, more than half a century ago: do all cells of an organism have the same set of genes? Can we reprogram a cell into another?

2.2. From experimental embryology to the regulation of the embryo 3D genome: ***Jacqueline Deschamps*** *(Hubrecht Institute, Utrecht, NL)*

The regulation of genes that pattern the embryo has been the theme of Jacqueline Deschamps research for the last 30 years. Hired by Siegfried de Laat, she has dedicated her career to understanding the molecular control and the mode of action of a family of transcription factor-encoding genes (Hox and ParaHox genes) in growth and morphogenesis of the mouse embryonic axis.

In the first part of her presentation, Jacqueline Deschamps recapitulated the history of the Hubrecht Institute (now summarized in the introduction of this report). She went on to show how her research lines have continued the Hubrecht tradition in developmental biology, as they were initially linked to work of Pieter Nieuwkoop and Kirstie Lawson, an embryologist who worked in the Hubrecht lab between the early 60 s and 2004.

Jacqueline then presented two ongoing studies. The first one concerns the growth modalities of the developing mouse embryo. While the anterior embryonic tissues are laid down early from the epiblast and the earliest mesoderm, possibly in a way comparable with the way the Xenopus embryo develops and undergoes mesoderm and neural induction, the more posterior tissues depend on the contribution of axial neuro-mesodermal stem cells first discovered in the lab of Valerie Wilson in Edinburgh.

The dichotomy between morphogenesis of anterior and posterior embryonic tissues is reflected in the phenotype of mutants studied in the Deschamps lab. Since her initial characterization of the requirement of Cdx (paraHox) genes for completion of embryonic axial extension, she and her colleagues have unraveled the molecular genetics behind this essential function of these Hox family transcription factors (Young et al., 2009). They also discovered that these genes play a crucial role in maintaining the niche of the axial stem cells by sustaining active growth signaling by the Fgf and Wnt pathways (Bialecka et al., 2012; Neijts et al., 2014). Recently they utilized genomic tools like chromatin immunoprecipitation and sequencing (ChIP-seq) to pinpoint the molecular interactions involved. They showed how the Cdx pathway intersects the pathway of another essential gene product, T Brachyury, to drive post-occipital tissue growth, and how Hox/Cdx factors orchestrate slowing down and ultimate arrest of axial elongation.

The second study aimed to identify the trigger and the control mechanism of the sequential turning on of the clustered Hox genes. Those are evolutionary conserved key genes for the acquisition of identity by embryonic tissues. This study was initiated in collaboration with Kirstie Lawson, when they found that the early expression of Hox genes in the primitive streak of the early mouse embryo expanded in response to inductive processes rather than reflecting the spread of cell

lineages (Forlani et al., 2003).

Recent work in the Deschamps lab continued tackling the beginning of this process. The very first Hox gene transcription in the early epiblast was recently found to be set up by Wnt signaling. Using epiblast stem cells as a model that they validated for the early embryo, they could identify a number of transcriptional enhancers, some of which were Wnt responsive, in the 3′ (early) side of the HoxA cluster (taken as a paradigm). The relatively open chromatin at these 3′ enhancers poises the specific activation of the cluster on that side, a puzzling observation until then.

Using recent genomic and epigenetic technologies (chromatin conformation capture or 4 C, ATAC-seq and ChIP-seq for histone marks), they discovered that several successive 3′ polarized epigenetic events underlie the early priming of this 3′ region for expression (Neijts et al., 2016). This explains at the molecular level the observations obtained ten years earlier using classical techniques of embryonic tissue explants and recombinants (Forlani et al., 2003). This early tropism prepares the constructive interactions between the 3′ regulatory region and the earliest HoxA genes via the newly discovered enhancers, all of which were confirmed to be active in embryos.

The recent advance in these particular studies illustrates the distance covered in the Hubrecht Institute in the study of most fascinating issues in developmental biology: from using classical approaches of embryology to adopting the recent cutting edge genomic techniques.

The molecular genetics of the initial and sequential turning on of the Hox genes, is also the subject of a review in the present issue of Developmental Biology (Neijts and Deschamps, this issue).

2.3. *From experimental embryology to human pluripotent embryonic stem cells.* ***Christine Mummery*** *(Leiden University, NL)*

The interest of the Hubrecht laboratory in stem cells research was initiated by Siegfried de Laat when he was still a group leader in the Hubrecht lab, a few years before he became director.

Christine Mummery joined his lab as a postdoc in 1978 and started focusing on embryonic carcinoma cells. These were first discovered in 1954 in mice that developed a tumor called teratocarcinoma (a malignant germ cell tumor) with high frequency in the testis. These teratocarcinomas were used to isolate a stem cell population called embryonic carcinoma (EC) that could be grown and differentiated in culture. EC cells could also take part in normal development in chimeric mouse embryos but were not able to contribute to the germ line. However, they formed a paradigm for embryonic stem (ES) cells that were derived directly from the inner cell mass of mouse blastocysts in 1981 by Evans and Kaufman (Evans and Kaufman, 1981). These ES cells did possess the ability to generate derivatives of the three germ layers in chimaeric embryos, as well as primordial germ cells.

In 1998, James Thomson and colleagues reported methods for deriving and maintaining human embryonic stem (hES) cells, from the inner cell mass of human blastocysts that were produced through in vitro fertilization and donated for research (Thomson et al., 1998). These studies were performed in the US, but a second study in Australia followed 2 years later (Reubinoff et al., 2000). Given the lack of legislation in the Netherlands to allow the isolation of new hES cell lines, Christine Mummery, then a group leader of the Hubrecht lab, flew to Australia and brought back the first hES cells in the country.

It was only in 2002 that her efforts together with those of Siegfried de Laat and Jan Sixma resulted in the first Netherlands Embryo Law, supported by minister E. Borst. Under this law, it was possible to derive hES cells for research provided 1) that there was no alternative, 2) that the embryos were surplus from IVF clinical attempts (thus had not been generated specifically for research), and 3) that proper permission from both gamete donors was obtained. The Hubrecht lab derived a total of 4 hES cell lines that became the focus of pluripotency and differentiation studies in vitro. The Mummery group devoted later efforts to the differentiation into cardiomyocytes and understanding how their fate could be directed in vitro (Passier et al., 2008).

A major breakthrough took place a few years later. Inspired by Sir John Gurdon's cloning experiments in *Xenopus* showing that cells in late stages of development can be reprogrammed and return to an embryonic state: Shinya Yamamaka demonstrated that mouse and human somatic cells could be reprogrammed to pluripotency simply by transfection of only 4 transcription factors and in this way created “Induced pluripotent stem cells” (iPS cells). This work was rewarded by the Nobel prize in Physiology or Medicine in 2012 (Lensch and Mummery, 2013) and led to important innovations in personalized or patient derived models for cardiac disease in humans (Bellin et al., 2012).

Christine Mummery's research now largely makes use of hIPS cell lines, not for transplantation of cardiomyocyte derivatives to repair the damaged heart, but to understand cardiac diseases, including arrhythmias, heart failure and associated cardiomyocyte defects. The aim of her group is to design and test drugs to cure or treat these conditions. Using cardiomyocytes differentiated from hIPS cells to screen for small molecules able to slow heart beat rate, drugs (already approved by the American FDA for treating other conditions) have been shown to be really potent in treating arrhythmias. This is what is known as drug repurposing.

She then described studies by American scientists using a similar strategy with hIPS cells differentiated into motor neurons to identify drugs able to treat amyotrophic lateral sclerosis (ALS), a dreadful neurodegenerative disease. They succeeded in identifying a drug able to increase motor neuron viability in two independent cases of familial ALS. These molecules are now being tested in clinical trials for sporadic forms of ALS. This illustrates the power of hIPS cells in disease modeling and identifying drugs that can be used in the clinic.

3. The genomic era

3.1. *From RNAi to Ziwi: The transgenerational effects of small RNAs.* ***René Ketting*** *(Institute of Molecular Biology, Mainz, DE)*

René Ketting is the archetype representing the genomics turn that the Institute took at the end of the 20th Century. He originally trained as a chemist and (he says) only discovered Developmental Biology when he joined the group of Ronald Plasterk at the Hubrecht. He remained as a group leader until 2011 when he was hired as a director of the Institute of Molecular Biology in Mainz, Germany.

René Ketting started working on the gene-silencing pathway of RNAi in C.elegans, and he noticed that when transposons are removed, the animals are defective in RNAi. Transposons are small sequences representing up to 50% of the eukaryotic genome. They have a tendency to transpose (hence their name) and create genomic instability. However, some of these elements are crucial for germ cell development (de Albuquerque et al., 2015).

In RNAi, double stranded RNA (dicer dependent) and small interfering RNA (dicer independent) converge on a protein called Argonaute (Ago) that binds the small piece of double stranded RNA to target the mRNA to be silenced. Members of the Ago family are the PIWI proteins that bind piRNAs in the germline in a dicer independent manner.

In zebrafish, two PIWI proteins can be identified, named ZILI and ZIWI that are specifically expressed in both male and female germ cells (Houwing et al., 2007; Roovers et al., 2015). They specifically bind 26–30 bp piRNAs that are produced from all types of transposons (Luteijn and Ketting, 2013). Unlike in the RNAi pathway, when PIWI proteins cleave a target, the resulting RNA fragments are not simply degraded, but instead can be used to generate new piRNAs, leading to higher levels of piRNAs when a transposon is more strongly expressed. Overall, piRNA levels reflect transposon activity.

René Ketting outlined three big questions in the field: 1) How are

transposons identified; 2) How are transposons controlled; and 3) Does this affect the rest of the genome? To answer these questions, the Ketting lab developed a zebrafish system where GFP is being targeted by piRNAs. Using this system, he could show that piRNA activity in zebrafish seems very much restricted to the germ cells and happens mostly at the post-transcriptional level. This is in contrast to many other animals where PIWI proteins and piRNAs also target chromatin, in order to silence transposons at the transcriptional level.

Interestingly, the level of piRNAs that target GFP responds to target RNA availability in what seems a strongly non-linear fashion, whereby a slight increase in target RNA results in a strong increase in piRNA level. Non-linearity can also be observed at the phenotypic level, i.e. the level of GFP expression directly. Either GFP appears to be fully silenced, or it is active, with little in-between scenarios. This strongly suggests that piRNA systems may work with thresholds, rather than with gradual silencing mechanisms.

Using the GFP-piRNA system, the Ketting group also demonstrated that the maternal deposition of piRNAs into embryos is essential for setting up stable GFP silencing. Embryos that have all the required loci for GFP piRNA production, but lack maternally provided GFP piRNAs are not able to establish effective GFP silencing, suggesting that during early development the maternal piRNA pool is used to shape zygotically expressed piRNA repertoires. Evidence for this has also been found in relatively distant zebrafish strains, where again maternal dominance on piRNA profiles is apparent. It is unknown how maternal piRNAs exert this function of influencing zygotic piRNA biogenesis.

Finally, René Ketting demonstrated that maternal deposition of piRNAs is not restricted to zebrafish, but also happens in mammals. In particular, he showed that bovine embryos contain a specialized PIWI protein, named PIWIL3 that is produced in late stage oocytes and binds to transposon-derived piRNAs. These findings suggest that maternal influence on embryogenesis through small RNAs is an evolutionary well-conserved phenomenon that thus far has remained fully untouched in mammals.

4. Bridging to cancer biology

4.1. Cell fate decisions after DNA damage. ***René Medema (****NKI, Amsterdam, NL)*

Progress in the understanding of developmental biology is often argued to lead to understanding of cancer. Cancer is possibly the most studied disease in modern bio-medical research, and DNA damage (induced by oxygen radicals, UV, toxic compounds (including chemotherapy) and irradiation) is one of the major causes of cancer. It is therefore critical to understand the fate of cells after such damage.

René Medema, a UMC Utrecht professor, now scientific director of the Nederlands Kanker Instituut (Dutch Cancer Institute) and chairman of the Scientific Advisory Board of the Hubrecht, has spent a large part of his career studying the fate of cells after exposure to DNA damage.

DNA damage is sensed by a “DNA damage checkpoint” that leads to an arrest of the cell cycle to allow repair: If the damage can be fixed, the checkpoint response is reverted; the cell recovers and restarts its progression through the cell cycle. Sometimes, the cell escapes the checkpoint and continues to divide with damaged DNA, leading to mutagenesis and propagation of these mutations to the daughter cells, possibly leading to tumor formation. When the damage is excessive and cannot be repaired, this can lead to exit from the cell cycle, senescence or even cell death by apoptosis. The Medema group is trying to resolve what determines cell fate in terms of cell exit from the cell cycle, damage repair and recovery, or permanent arrest and cell death.

DNA damage checkpoints are both active in the G1 and G2 phase of the cell cycle. Using the FUCCI system to easily detect cells at specific phases of the cell cycle, the Medema group evaluated the ability of cells in G1 and G2 to recover after DNA damage (using 4 Gy irradiation). He showed that while cells in G1 remain competent to recover for several days, the recovery competence of cells in G2 is lost within several hours. Thus, the decision to recover and re-enter the cell cycle, or to exit the cell cycle and become senescent is taken in the first few hours following the damage. This rapid decision is entirely dependent on the tumor suppressor p53, and the G2 arrest remains reversible when p53 is mutated.

How does this work? In non-damaged cells, the essential trigger for mitotic entry is nuclear cyclin B1. Upon phosphorylation of cyclin B1 by CDK1, cyclin B1 enters the nucleus and triggers the onset of mitosis. Upon DNA damage, there is a rapid but transient activation of p53 in G2 cells. This, in turn, results in the induction of p21, a p53 effector gene. High levels of p21 cause massive translocation of cyclin B1 to the nucleus and depletion from the cytoplasm, irreversibly leading to cyclin B1 degradation, and permanent cell cycle arrest. In cells with low or intermediate levels of p21, a pool of cyclin B remains in the cytoplasm, and the arrest is fully reversible.

Medema's group also noticed that a small subset of G2 cells previously characterized as “antephase” cells are hypersensitive to DNA damage and are immediately removed from the proliferative cycle to protect genome stability, even at very low levels of DNA damage. He showed that this response is caused by the loss of Emi1 in antephase, and furthermore showed that checkpoint reversibility in G2 strictly depends on Emi1.

4.2. Cell signaling by receptor tyrosine kinases; from basic principles to cancer therapy by ***Joseph Schlessinger,*** *Yale university, CT*

The human genome contains 89 protein tyrosine kinases. 58 are receptor tyrosine kinases (RTK) integral to the plasma membrane and 31 are cytoplasmic. Importantly, more than 50% of the RTKs are mutated or aberrantly expressed in cancer. Developing protein kinase inhibitors might therefore have a huge benefit to fight the disease. 29 of these inhibitors are FDA approved and 400 are currently in clinical trials. Joseph Schlessinger from Yale has had extensive collaborations with Hubrecht researchers, including Siegfried de Laat, Wouter Moolenaar and Jeroen den Hertog. Together with Axel Ullrich, he is the founder of the Sugen company that has developed two successful FDA approved cancer drugs designated Sutent and Crisotinib.

The concept behind the activation of protein tyrosine kinase receptors at the plasma membrane is that most of them normally exist as inactive monomers that are in steady state with either inactive or active dimers. These are then stabilized through binding to their ligands that stimulate both RTK dimerization and activation. This is the case of the three RTKs described below (Lemmon and Schlessinger, 2010).

The KIT dimerization motif has been mapped to D4, a small region in the extracellular domain (Reshetnyak et al., 2015; Yuzawa et al., 2007). The formation of the D4/D4 interface could therefore be antagonised by blocking antibodies or inhibited by small molecules. This is especially critical because in cancer, specific mutations can lead to the stabilization of active dimer in a ligand-independent manner. This is also the case for KIT where oncogenic mutations in D5 lead to stable dimer formation. This is due to the salt bridge affinity in the D4-D4 interaction that increases by 200–500 fold, making the receptor constitutively active. These mutations are prominent in gastro-intestinal cancer, and in subtypes of melanoma or leukemia among other cancers. The Sutent cancer drug that targets the D4-D4 interface has so far been applied for treatment of 250,000 patients in 109 countries.

BRAF V600E is a mutation found in approximately 50% of metastatic melanomas and 30% of thyroid carcinomas and 4% of most solid tumors The BRAF inhibitor PLX4032 (Zelboraf) has been crystallized with BRAF and when administered in patients, it leads to a spectacular shrinkage of the tumor, which is evident by visual inspection and by assessment of the metabolic activity of the tumor (Warburg effect). However, cases of resistance occur mostly because of negative feedback mechanisms via NRas and other components of the Ras/MAPK pathway.

ErbB3 plays an important role in many cancers and it interacts with ErbB2, which is often overexpressed in breast cancers. ErbB3 also forms heterodimers with EGFR as well as with ErbB4 in response to stimulation by EGF, TGF-alpha and neuregulin Nrg1 and 2 that leads to a strong survival pathway. The crystal structure of ErbB3 reveals that its extracellular domain is maintained in an auto inhibited state by intramolecular interactions mediated by contacts between domain 2 and domain 4. The inactive monomeric auto inhibited tethered state of ErbB3 exists in steady state with an open extended configuration of the extracellular domain. This open conformation is poised toward formation of activated homodimers or heterodimers with other members of the EGFR family, including ErbB2 or ErbB4. An inhibitory therapeutic antibody designated KTN3379 exerts its inhibitory activity by preventing the hinge movement necessary for both receptor activation and for high affinity ligand binding. KTN3379 is now in phase I trial, offering opportunities of new treatments.

This presentation made a strong case for crystal structures being instrumental into the design of novel and specific inhibitors that are applied for cancer treatment.

5. Bridging to adult stem cells and regenerative medicine via Wnt

5.1. Wnt and liver stem cells. ***Roel Nusse****, Stanford University, CA*

The transplantation experiments of Hans Spemann and Peter Nieuwkoop (see above) in the last century led to the concept of "embryonic induction" whereby one region of the embryo communicates with its adjacent part and determines its development. Transplantation experiments revealed that this communication occurs via the release of a secreted factor in the posterior part of the embryo that later turned out to be Wnt. In fact, posterior Wnt signaling operates in all animal phyla leading to directional growth and axis formation in the embryo. Remarkably, a similar principle occurs in adult tissue stem cells that are also activated by Wnt signaling.

The field of Wnt signaling was briefly reviewed by Roel Nusse (a Dutch national who has spent most of his prestigious career at Stanford University California and still is a frequent visitor and colleague of the Hubrecht Institute). Roel Nusse has worked on Wnt for most of his career and discovered many genes, including Wnt itself, and genes of the so-called "canonical Wnt pathway". He and his group also have contributed largely to the knowledge to the role played by this pathway in development and in stem cell biology.

Roel Nusse presented his more recent work on the role of Wnt in liver regeneration. Until recently, liver stem cells were not known to exist and all hepatocytes were thought to proliferate at low rate. However, Nusse and his group found that a specific group of hepatocytes, those around the central vein (the pericentral hepatocytes), are the only ones responsive to Wnt (Hu et al., 2007). They are also the only hepatocytes to proliferate and were proposed by the Nusse group to act as liver stem cells.

This was demonstrated by lineage labeling where pericentral hepatocytes are specifically marked by tagged Axin2 (also by Lgr5, another Wnt target gene, see below by Nick Barker). Upon tamoxifen activation of the tracers' expression, many cells in the liver lobule were seen to have originated from the peri-central area. Although cell turnover is much slower than in the small intestine (see below), this shows that the pericentral hepatocytes act as stem cells to maintain liver homeostasis.

But where does the Wnt signal come from? The endothelial cells of the central vein secrete Wnt9b and Wnt2, and Wnt expression in the vein is essential for clonal expansion (Wang et al., 2015). The central vein epithelium is therefore proposed to form a niche for the pericentral hepatocytes that act as stem cells.

In contrast to the majority of hepatocytes in the liver that are polyploid, 95% of pericentral hepatocytes are diploid. Roel Nusse also showed that the pericentral hepatocytes express the stem cell marker Tbx3 and accordingly, Tbx3-/- mice do not have a liver. Tbx3 is expressed in the embryonic anlage of the liver at E9.5/E10.5, and in the liver around embryonic day 14.5/15.5, before being confined to the pericentral vein cells in the adult.

In conclusion, liver stem cells (the pericentral hepatocytes) are responsive to Wnt, a feature common to most stem cells, except muscle satellite cells. There are, however, a number of tissues for which stem cells have not yet been identified, such as the pancreas and the heart, and these are areas of intense research at the Hubrecht.

5.2. Lrg5-expressing stem cells in the stomach. ***Nick Barker****, A*Star Institute of Medical Biology, Singapore*

Nick Barker was a postdoc in the Clevers lab when, in 2007, they discovered the Wnt target gene Lgr5 as a marker of stem cells in the intestine. Looking for genes differentially expressed in response to Wnt signaling in colon cancer cells, Lgr5 appeared on top of the list. In the healthy intestine, Lgr5 expression was restricted to thin columnar cells intercalated between the Paneth cells at the base of the intestinal crypts. These poorly characterized cells, known as Crypt Base Columnar (CBC) cells had previously been proposed as intestinal stem cells, but there was no evidence to support this unpopular theory. In vivo lineage tracing using an Lgr5-GFP-ires-CreERT2 knock in mouse was therefore used to investigate the stem cell properties of these Lgr5-expressing CBC cells. Marked Lgr5-expressing CBC cells at the crypt base were shown to generate entirely labeled crypts 15 days later, identifying the Lgr5-expressing cells as multipotent intestinal stem cells (Barker et al., 2007). Lgr5 was later found to mark stem cells in a range of tissues, including the skin, stomach and kidney (Leushacke and Barker, 2012; Ng et al., 2014).

Following his move to Singapore, Nick Barker switched focus to study stomach stem cells, with the aim of understanding their roles in maintaining the stomach lining and in driving stomach disease (Barker et al., 2010). In Asia, stomach cancer is still highly prevalent due to the spread of the inflammation-causing Helicobacter infection through poor sanitation. As long-lived cells, stomach stem cells are considered a likely source of stomach cancer following mutation (Barker et al., 2009).

The glandular region of the stomach comprises the pyloric antrum connected to the small intestine and the acid-secreting corpus region responsible for food digestion. The glandular epithelium is organized into tubular glands, which, like the small intestinal epithelium, is constantly renewed throughout life by resident stem cells. Using the Lgr5 reporter mouse described above, the Barker group found Lgr5 expression to be largely confined to the base of the pyloric glands (equivalent to the crypt in the intestine). Using a similar lineage tracing strategy, these Lgr5-expressing cells were shown to be daily stem cells responsible the maintenance and repair of the stomach epithelium. Ablation of the Lgr5-expressing stem cells in the stomach in vivo using an Lgr5-DTR-GFP mouse model severely impaired epithelial renewal, highlighting the critical role of these cells in maintaining a healthy stomach lining. Following mutation of the Wnt pathway, these stomach stem cells also caused early stomach cancer to develop in mice.

With the aim to identify new stomach stem cell markers that can be used to isolate human stomach stem cells, expression profiling of FACS-sorted pyloric stem cells was performed and the expression signature compared to that of the Lgr5+ stem cells in the small intestine and colon. Several new stomach-specific stem cell markers were identified, including a membrane-expressed gene involved in water transport. This new marker was co-expressed with Lgr5 at the gland base, but not in the intestine. Antibody-based sorting of stomach cells expressing this marker was used to confirm their stem cell identity in organoid culture assays.

Absence of Lgr5 reporter gene expression in the corpus of the original Lgr5 KI model indicated that no Lgr5 corpus stem cells exist. However, using a new Lrg5 reporter mouse model (Lgr5-2A-CreERT2)

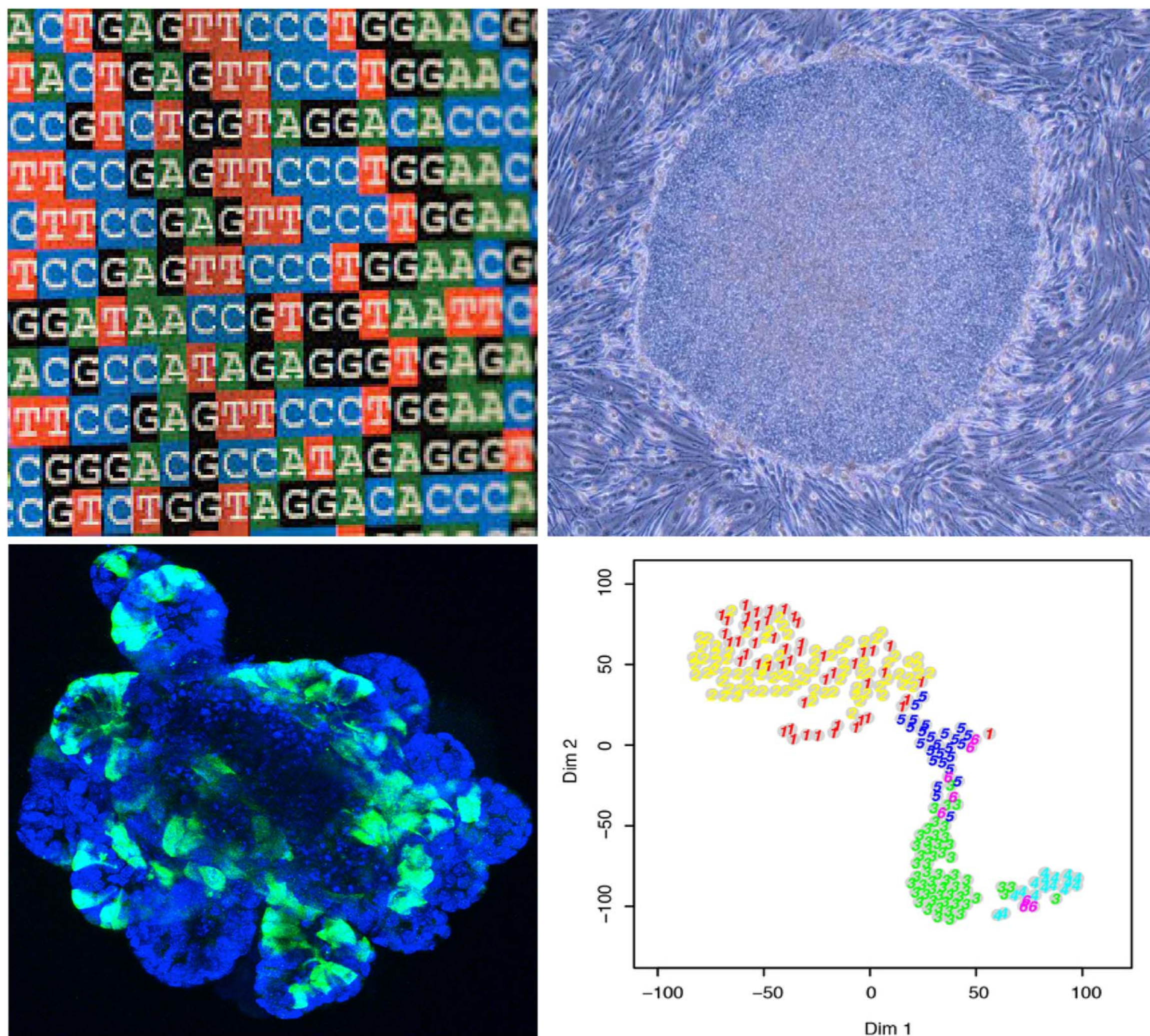

Fig. 2. From embryology to stem cells technologies. Illustration of sequencing (top left), stem cells (top right), intestinal organoid (bottom left) and RaceID representation from single cell sequencing (bottom right).

that circumvents reporter gene silencing arising in the original model due to loss of Lgr5 gene function, Lgr5 expression was found on a subset of non-dividing Chief cells at the corpus gland base. Using lineage tracing, these Lgr5-expressing Chief cells were not found to play a role in daily epithelial homeostasis, fitting with their known daily role as sources of digestive enzymes. However, following damage, these Lgr5+ Chief cells acquired stem cell functions to drive epithelial regeneration and cancer following mutation.

Using this new Lgr5 reporter model, Barker's group have also identified novel Lgr5+ stem cells in other organs, including the liver, mammary gland and esophagus.

Nick Barker also described a new stomach-specific Cre mouse model that can be used to selectively introduce mutations into the stomach epithelium. This model has been used to efficiently drive stomach cancer in mice, highlighting its value in generating accurate models of human stomach cancer to help better understand stomach cancer progression as an essential pre-requisite to developing more effective therapeutics.

5.3. The organoid revolution in regenerative medicine:Hans Clevers, Hubrecht Institute and UMC Utrecht, Utrecht, NL

The meeting was concluded by Hans Clevers, the former director of the Hubrecht Institute, former president of the KNAW, and present Director Research, Princess Maxima Center for Pediatric Oncology in Utrecht.

One of the major findings in the Clevers group was the discovery on the crucial role of the Wnt pathway in homeostasis of the small intestinal epithelium. This discovery came from Hans Clevers' investigations earlier in his scientific career. Trained as an immunologist focusing on T cell development, he cloned the T cell-specific transcription factor TCF1. TCF1 was originally thought to be lymphoid-specific, and is in fact expressed in a complex pattern during mouse embryogenesis. In 1996, together with Olivier Destree, Hans Clevers discovered that a Xenopus member of the TCF/Lef family interacts with beta catenin that was known to be at the cell-cell junction but critically, is also involved in embryonic axis formation. They showed that the TCF/beta catenin complex was required for the transcriptional response to Wnt signals (Molenaar et al., 1996). The Wnt pathway was shown to be a key player in maintaining adult stem cells in the intestine and is constitutively active in APC-mutant human colon cancer. This made the search for Wnt target genes differentially expressed in the intestinal crypts a major goal of the Clever's lab. The Wnt target gene Lgr5 was identified and was shown later to be the receptor for R-spondin, a molecule that amplifies the Wnt signal (de Lau et al., 2011). In 2007, the Lrg5-knockin mouse that Nick Barker has generated (see above) allowed him demonstrate that Lgr5+ crypt cells are the stem cells of the small intestine and importantly, their maintenance depends on Wnt (Barker et al., 2007).

Their identification resulted in a clearer description of their features: Intestinal stem cells are not quiescent; they are not rare as

they represent up to 10% of the crypt and they do not divide asymmetrically. The stem cell hierarchy is not unidirectional: daughter cells at the boundary of the stem cell compartment can revert to Lgr5 positive stem cells upon damage of the crypt. Interestingly, Hans Clevers mentioned that the existence of intestinal stem cells had been proposed, and their properties predicted by the French researcher Charles Leblond in the 60ies (McGill, Montreal), who is considered as a stem cell pioneer (Leblond et al., 1967).

With pure FACS sorted Lrg5-GFP positive stem cells in hand, the next step was to culture them. Using a mixture of factors known to have positive effects on stem cells in vivo (R-spondin, EGF, Noggin for the BMP signaling), Toshiro Sato managed to grow a single stem cell into mini-guts, the intestinal organoids that comprise all the intestinal cell types. These organoids can then be cultured for years and they are genetically stable (Sato et al., 2011, 2009). The potential of these organoids is enormous. Already they are used in transplantation experiments and can rescue damaged colon epithelium in mice with the hope to soon do the same in human. Organoids are also generated from colon tumors and currently tested for their usefulness to screen cancer drugs for their efficiency in reducing tumor growth before they are administered to patients.

The Clevers lab has now generated organoids from nearly all internal organs, including lungs. The lung is a ciliated airway and the coordinated beating of the cilia is critical to clear the secreted mucus. The lung organoids reproduce this key feature and are being used to develop therapeutics strategies to cure cystic fibrosis. Inactivating mutations in the CFTR gene (encoding cystic fibrosis transmembrane conductance regulator) cause Cystic Fibrosis. As a result, chloride channels are dysfunctional, the mucus is not cleared away, leading to chronic inflammation.

CFTR lung organoids are being used to screen individual patients for their sensitivity to recently developed drugs that open the CFTR channels. These drugs are presumed to be highly mutation-specific. The test is simple as the opening of these channels leads to the swelling of the organoid. Several patients with uncommon CFTR mutations have already been identified that responded well in the organoid assay. They subsequently showed a good clinical response to the drug. Furthermore, the gene editing system CRISPR-Cas9 to repair the CFTR mutation in lung stem cells has succeeded, offering possibilities to re-inject the organoid-derived stem cells in the patients for clonal expansion (Schwank et al., 2013).

6. The future of the Hubrecht Institute. Alexander van Oudenaarden, Hubrecht Institute, Utrecht, NL

The inverted pyramide standing on Hubrecht's shoulders now houses and nurtures many disciplines. These range from developmental biology, cancer biology, cell biology, stem cell biology, and since 2012, also quantitative biology (Fig. 2). Research in this new direction is driven by present director, Alexander van Oudenaarden. It is expected that the excellence in basic research will continue at the Hubrecht for generations to come, likely to lead to deeper understanding of the molecular basis of biological processes and hence to better understanding of diseases and their treatment.

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