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Immunobiology of SCNT derived Embryonic Stem Cells: Foreign mitochondria as an immunological barrier

Dissertation

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1 Introduction

In December 1905 the early days of modern medicine began with the first reported tissue transplantation (cornea transplantation) performed in Olmutz, Moravia (now Czech Republic). Around 5 decades later the first milestone in organ transplantation had been established when a team of physicians in Boston, USA, performed the first successful kidney transplantation ever reported. Since these early days immune rejection of the transplanted tissue had been known, except when performed between identical twins. Due to extensive research efforts scientists have revealed that the differences in proteins structures between allogeneic (genetically not-identical) individuals trigger the rejection by an immunocompetent host. Large diverse groups of molecules involved in immune recognition were discovered and defined as histocompatibility antigens, divided into three groups 1) major histocompatibility complex (MHC) molecules, in humans: human lymphocyte antigen (HLA) 2) minor histocompatibility complex antigens (mHCs) 3) ABO blood group antigens (Tang and Drukker, 2011). Hence, even nowadays, an immunosuppressive therapy is still mandatory and is included in protocols when organs or tissues are transplanted between allogeneic individuals.

Since transplantation medicine started it became crucial to overcome the lack of available organs needed due to the high number of dying patients, waiting on the organ-lists all over the world. To overcome this hurdle a novel strategy was offered when embryonic stem cells (ESCs) had been first isolated (murine ESC in 1983 (Kaufman et al., 1983); human in 1994 (Bongso et al., 1994)) and also their adult partners, adult stem cells (ASCs) with the first bone marrow transplantation in a 2-year old patient with Wiskott Aldrich syndrome (Bach et al., 1968). Their directed differentiation into a variety of tissue specific cell types seemed and still seems very appealing for organ restoration and cell-based therapies. However, up to today a successful transfer of the therapeutic promises of ESCs into the clinic is absent. Undefined growth of ESCs leading to teratoma formation and immune rejection of transplanted ESCs are still major problems that need to be resolved before a successful clinical translation can be made. Therefore current medicine is not only focused on ESCs but rather on ASCs, since they are already tissue-specific and lack the ability to form a

teratoma. Nevertheless an allogeneic approach for ASCs-therapy leads to a rejection of the transplanted cells by an immunocompetent recipient.

1.1 Totipotency/Pluripotency/Multipotency

All mammalian life derives from the fusion of an egg cell and spermatocyte resulting in the zygote. During the progression to a fully living organism the cell proceeds from a state of totipotency, specialty of the zygote and blastomere, to cells that are limited in their developmental potential. *Totipotency* is used to describe cells that are not only able to differentiate into any of the three germ layers (mesoderm, endoderm, ectoderm), but also have the ability to form extra embryonic tissue, building together the fundament of an embryo. The rapid division of the zygote, also called embryonic cleavage, results in the state of the morula. After a fixed number of cell cycles, the outer cells of the embryo are determined to two lineages: one which will form the placenta, the trophoblast lineage; and the rest which will generate the epiblast and the hypoblast; the bipotential inner cell mass (Selwood and Johnson, 2006). At later stages of the development, the epiblast and the hypoblast form the embryo and the yolk sac, respectively (Hanna et al., 2010). These later stages are no longer totipotent, hence they loose the ability to form an embryo resulting in the state known as *pluripotency*. Since these cells build all cell lineages inside an embryo, they demonstrate a tremendous potential as the source for regenerative medicine and therefore are expected to have an enormous impact on regenerative therapy.

In contrast, cells that are derived from later stages of the embryo-development have lost their ability to form cell types of all three germ layers, since they already differentiated into a specific direction. But they can still serve as the progenitor of a variety of more specialized cell types. These properties define the multipotent status of adult stem cells. In clinical settings only adult stem cells are currently used due to their lack of teratoma formation, an out of questionable necessity for clinical approaches.

1.2 Self-renewal

Cells derived from various embryonic and postnatal stages, stem cells have the potential of self-renewal and the capacity for differentiation (Jaenisch and Young, 2008), but lack the power to form extraembryonic trophoblast lineage (Rossant, 2008). When they were first created, the inner cell mass of the embryo of a mice was explanted (Kaufman et al., 1983). When injected into mouse blastocysts, these murine embryonic stem cells (mESCs) reiterated full developmental potential, showing cells of all three germ layer. Coming from the inner cell mass, ESCs express key pluripotency genes such as Oct4, Sox2, Nanog, stage-specific embryonic antigen 3&4 (SSEA3, SSEA4) as well as TRA-1-81 and TRA-1-60 (Nagano et al., 2008, Nichols and Smith, 2009). These transcription factors maintain the pluripotent state of ESCs (Boyer et al., 2005). Nevertheless ESCs show different biological and molecular characteristics from their *in vivo* counterparts of the inner cell mass, making them distinguishable. For example, ESCs show unlimited proliferation potential, and their genome is highly methylated (Meissner et al., 2008) whereas cells from the inner cell mass are not self-renewing possessing a globally hypo-methylated genome (Santos et al., 2002).

The ability of self-renewal and pluripotency is granted for unlimited generations. Therefore, it has given ESCs a major advantage over adult stem cells, since they are generally tissue-specific. Paradoxically it is also their disadvantage, as said before, since their fast growing character and the pluripotency let them easily turn in undefined growth *in vivo*, rising tumors, called “teratoma”. By definition a teratoma is:

“A true neoplasm composed of a number of different types of tissue, none of which is native to the area in which it occurs. It is composed of tissues that are derived from three germinal layers, the endoderm, mesoderm and ectoderm.”

(Gowindan, 2012)

These teratomas are known to occur after ESC transplantation in animal models (Koch et al., 2008, Swijnenburg et al., 2008). Nevertheless, teratoma formation after ESC transplantation is nowadays the *in vivo* confirmation

method for pluripotency of ESCs since they consist, by definition, of cell types of all three germ layers.

1.3 Immunobiology of Embryonic Stem Cells

1.3.1 Acute cellular immune response

Classic MHCI molecules are expressed by nearly all nucleated cells within the mammalian body, presenting processed cellular and extra-cellular proteins to circulating T-cells. Inside the human body the MHCI molecule consists out of the so-called HLA A, B or C-chain connected to a β2-Microglobulin. In mice the murine H-2 region of chromosome 17 encodes the complex's heavy chain. It can be divided into the classical I-a and non-classical I-b MHC gen. The classical MHC-Ia comprises H-2D, H-2K and H-2L subclasses, whereas the non-classical MHC-Ib contains H-2Q, H-2M and H-2T subclasses. One molecule of the MHC-Ib molecule is Mta. It consists of three gene products: M3a, a class I-b MHC heavy chain, Mtf, the N-terminus of the mitochondrial encoded ND1 subunit, and the β2-microglobulin (Vyas et al., 1992). MHC molecules are highly polymorphic and, in mice, are designated by a small letter (a, b, d, k, q, s etc.)

The interaction of the T-cell receptor and the MHCI molecule is one of the fundamental mechanisms to distinguish between self and foreign leading to rejection of transplants as seen in clinical organ transplantations, since the MHCI itself is highly immunogenic. After transplantation of an allogeneic organ, immunosuppression is obligatory leaving the patient with the complications normally accruing after immunosuppression, as for example higher infection rate, unwanted pharmacological side effects and possible tumor formation. Therefore, therapeutic strategies involving cells that are not detected by the host's immune system is a long lasting dream of many scientist and physicians. It was long believed that embryonic and adult stem cell are ignored by an immunocompetent host, since they under express MHC class I/HLA molecules, letting them be undetectable for allospecific CD8+ cytotoxic T-lymphocytes (cTLs) (Tian et al., 1997, Draper et al., 2002, Drukker et al., 2002). Despite these findings ESCs were universally rejected in an allogeneic/xenogeneic

setting, allocated by a severe infiltration of various types of immune cells involved in both innate and adaptive immunity 5 days after transplantation (Swijnenburg et al., 2008) [Figure 1]. Further analysis revealed a higher presence of both CD3+ T cells and B220+ B cells suggesting a distinctive role of the adaptive immune system in the transplant rejection of ESCs. An explanation for this illegitimate result could be that ESCs substantially up regulate their MHCI levels when treated with Interferon-γ (IFNγ), a pro-inflammatory cytokine highly secreted by T-helper (Th)-1 cells. Additionally a 10 fold-increase in MHCI expression on the cell membrane was seen when ESCs were directed into differentiation induced by aggregation using embryoid body (EB) formation. IFNγ has been even more effective forcing ESCs close to somatic levels of MHCI, which is above an 100-fold increase (Drukker et al., 2002).

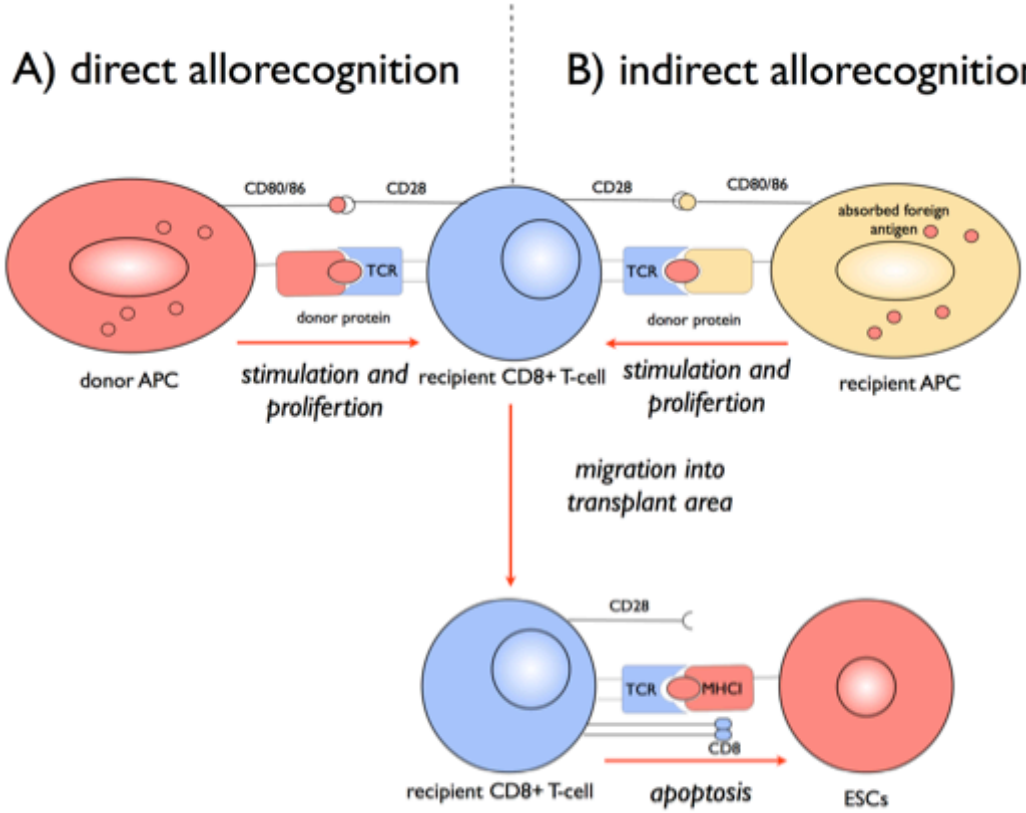

Figure.1 Direct and indirect allorecognition pathways. A) Direct allorecognition: T-cell respond to direct antigen presentation of donor-derived antigen presenting cells (APC). Next to determinants on the MHCI molecule, allogeneic structures found on the bound protein act as T-cell stimulants; B) Indirect allorecognition: Recipient APCs process peptides derived from allogeneic donors, primarily MHC molecules and present them in self-MHC to recipient T-cells. Co-stimulatory interactions between CD80/86 (B7) molecules with the CD28-counterparts on T-cells are needed to stimulate and propagate allospecific T-cells. Alloreactive T-cells recognize donor cells (here ESCs), which are driven into apoptosis. (TCR = T-cell-receptor)

In contrast, MHCII molecules have not been found on the cell surface of ESCs. MHCII are expressed constitutively by antigen presenting cells like macrophages, dendritic cells (DCs) and B-Cells and can be induced by cytokines (e.g. IFNγ). Still surface expression of MHCII-molecules on ESCs are not inducible by IFNγ (Drukker et al., 2002). However, upon ESC differentiation into hematopoietic and DC lineage showed an increase of these molecules (Slukvin et al., 2006, Senju et al., 2007).

To summarize, MHCI expression of ESCs increases upon differentiation and in an inflammatory milieu, present after transplantation. Therefore, the immunological immaturity of ESCs can be doubted since MHCI is present and contributes to their immunogenicity.

At the same time it is important to note that ESCs lack costimulatory molecules such as CD80 and CD86 (Drukker et al., 2006, Deuse et al., 2011a, Deuse et al., 2011b). Therefore ESCs might only weakly stimulate alloreactive T-cells. So the cytotoxic effect of T-cells might be decreased as well as third-party allogeneic dendritic cell-mediated T-cell proliferation (Li et al., 2004) if T-cells are only primed through ESC interaction alone. It must be highlighted that there are major differences in the immune biology of human versus murine stem cells, which can partly explain different outcomes.

ESCs transplanted in allogeneic or even xenogeneic recipients are usually infiltrated by various immune cell types and are eventually rejected, if the study was designed with a sufficiently long observation time. (Kofidis et al., 2005, Swijnenburg et al., 2005, Nussbaum et al., 2007, Robertson et al., 2007, Deuse et al., 2011a) This simple scenario was challenged, by observing the fate of variable doses of transplanted mESCs in allogeneic and syngeneic recipient mice. In allogeneic mice $1*10^6$ ESCs did not lead to teratoma formation, as expected. When $5*10^6$ ESCs 30% of the allogeneic recipient developed a teratoma. $20*10^6$ ESCs gave rise to teratomas in 90% of the allogeneic mice, indicating that the cell number applied probably overextended the immune system. However, the rapid and steady growth of the tumor forced the scientist to euthanize the mice after 3 to 4 weeks excluding the possibility to spot a

possible immune response that might have eventually controlled the tumor growth. Supporting, all allogeneic tumor showed an infiltration by CD3+ T-cells.

In summary these data highlight the importance of basic research to understand the immunobiology of stem cells since. Entire experimental setting, e.g. methods used for the evaluation of t-cell targeting, the use of human or murine ESCs, culture conditions, the amount of transplanted cells, and the end-point parameters of the study, have enormous effects on the outcome. However, the historical belief that ESCs are immunologically immature and can escape the recipient's immune system should be critically questioned. Nevertheless, a complete understanding of the interaction between the host's immune system and the transplanted ESCs is required before ESC-based therapy will have its place in regenerative medicine.

1.3.2 Humoral immune response

On of the major pathways to activate the humoral immune system is via IL-4 secretion of Th-2 cells. IL-4 stimulates B-cells proliferation as well as B-cell antibody class switching [Figure 2].

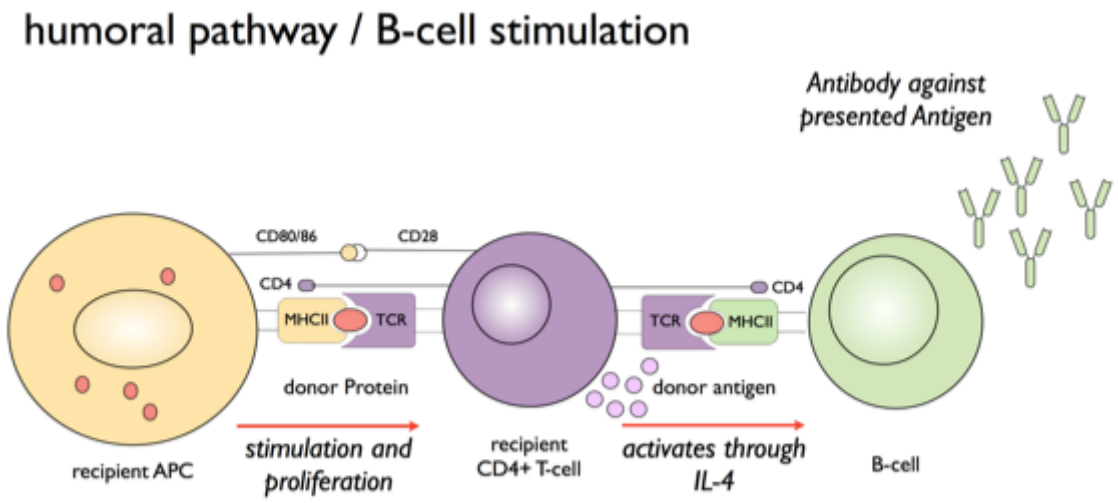

Figure.2 Humoral Pathway against transplanted cells: As in indirect allorecognition, recipient APCs process foreign proteins and present them in MHCII molecules to circulating CD4+ recipient T-cells. After stimulation and propagation, CD4+ T-cell secrete IL-4 to stimulate donor specific B-cells. Proliferation as well immunoglobulin class switching is initiated. B-cells are starting to secrete donor specific antibodies (DSA).

When undifferentiated human ESCs were injected into CD4+ T cell knockout or into CD8+ T-cell knockout mice, it was reported that the hESCs survived significantly longer in the CD4+-KO compared to the CD8-KO mice using the Bioluminescence Imaging (BLI). For BLI cells are stably transduced to express firefly luciferase, which allows cell type specific non-invasive longitudinal *in vivo* cell tracking by assessing of photon emission. An important advantage in using BLI is that only living cells express the expression of the fLuc reporter gene, which is integrated into the DNA of the transplanted cells. Therefore, longitudinal non-invasive *in vivo* cell tracking by assessing photon emission is possible. (Cao et al., 2005). In these studies, a significantly higher presence of donor- reactive antibodies against hESC was reported, compared to wild-type animals (Swijnenburg et al., 2008). Nevertheless this study showed that eventually both, CD4+-KO and CD8+-KO mice finally rejected their hESC xenograft. Another group demonstrated that systematic depletion of CD4+ T cell using anti-CD25 antibodies resulted in mESC engraftment in immunocompetent mice (Lui et al., 2010). Even though more data are needed to critically understand the underlying mechanisms, the data suggest a significant role of Th2-cell mediated immune response, pushing the adaptive immune system further into the spotlight.

1.3.3 Innate Immune system

The most prominent cell-population within the innate immune system are natural killer cells (NK cells), which are well known to lyse cells that under express Immunoglobulin Superfamily Cell Adhesion Molecules (Oldham, 1983), accordingly to the missing self hypothesis (Ljunggren and Kørre, 1990). This is normally observed when cells are transfected by viruses. The stimulus for NK cells to lyse target cells is mediated by an interaction of stimulatory and inhibitory signals represented by the NKG2, KIR (inhibitory), and KAR (activating) family of receptors (Raulet, 2006). MHCI-molecules as well as the HLA particularly support the inhibition of NK cells. Therefore, an under expression of these inhibitory receptors (as seen in ESCs) should render these cells more susceptible for NK cells since the equation would now be shifted towards the stimulatory site [Figure 3]. Some data demonstrate a sensitivity of hESCs derivatives to NK cell-mediated cytotoxicity (Preynat-Seauve et al.,

2009). However, it has been shown in several studies that hESC (Drukker et al., 2002) and mESC (Bonde and Zavazava, 2006, Dressel et al., 2008, Koch et al., 2008, Frenzel et al., 2009) lines are not or very weakly susceptible to lysis by NK cells in vitro. Differences in the degree of NK cell-mediated ESCs lysis can be explained by disparities in experimental conditions, use of various NK cell subtypes, as well as their activation level.

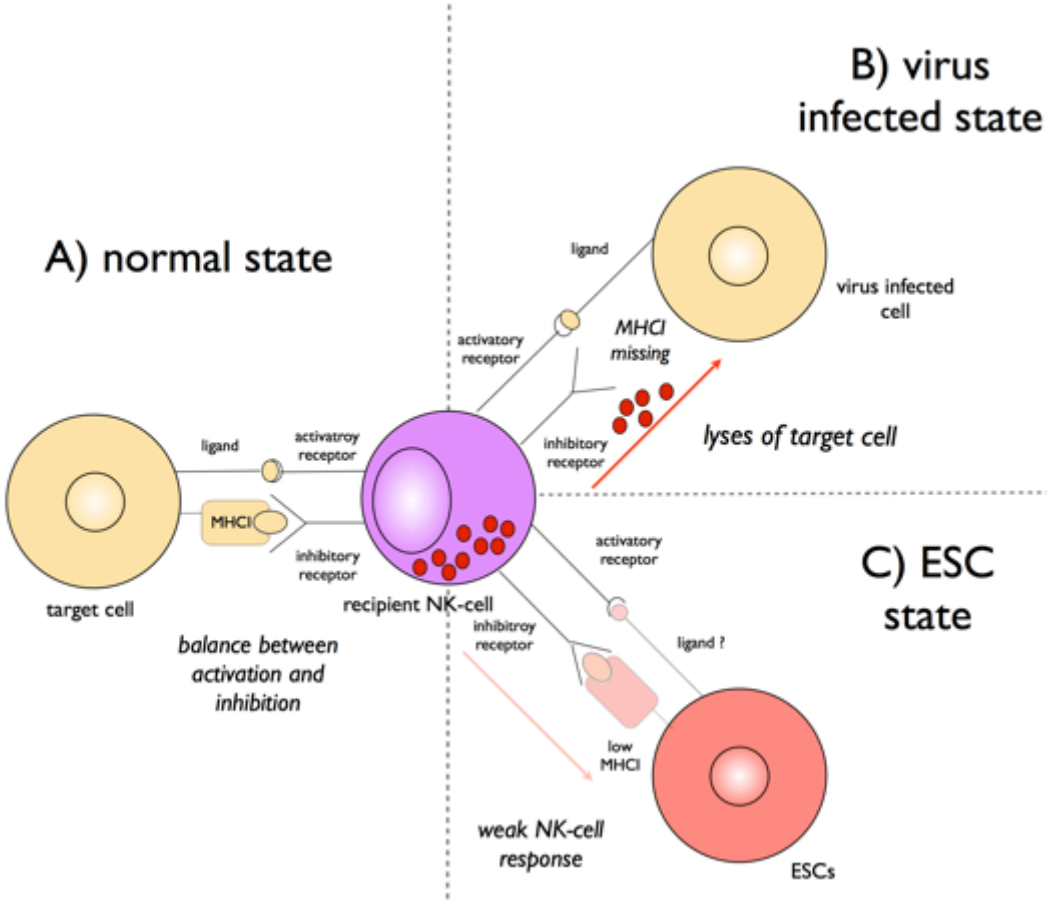

Figure 3. NK cell stimulation and inhibition pathways. A) Normal state: NK cell stimulation and inhibition is balanced due to both inhibitory and stimulatory signals represented by NKG2, KIR (inhibitory) and KAR (activatory) family receptors; B) Virus infected state: NK cells are well known to lyse cells that under express Immunoglobulin Superfamily Cell Adhesion Molecules, according to the missing self hypothesis; C) Since ESCs express low levels of MHCI molecules but lack activatory ligands, they are most likely weakly susceptible to NK-cells

However, “missing self” antigens are by far not the only prerequisite for NK cell-mediated lysis. Additionally, target cells need to express stimulatory ligands for NK cell activation as well as intercellular adhesion molecules (Yokoyama, 2005). By blocking the NKG2D receptor with a specific monoclonal antibody, it was shown that NK cells or are crucially reduced in their capacity to lyse different mESCs lines. By additional blocking of ICAM-1 cell adhesion the cytolysis of the mESC lines was abrogated (Tian et al., 1997). Indeed, it was demonstrated that

expression of stimulating molecules for NK-cell activating ligands is very low or below detection level on hESCs (Drukker et al., 2002). Still there has been evidence that allogeneic mitochondrial DNA can lead to a rejection process mediated through NK-cells (Ishikawa et al., 2010). However, more studies are needed to fully understand the balance of inhibitory and stimulatory molecules of ESCs to draw further conclusion in their immunogenicity towards NK cells.

1.3.4 Minor histocompatibility antigens (mHC)

Clearly, ESCs express mHC, mainly derived from mitochondrial DNA (mtDNA) and from Y-chromosome genes (H-Y antigens). However, all polymorphic proteins (differences in recipient and donor) should be considered “immunogenic”. Like other self-peptides, mHC, are derived from the proteolysis of endogenous proteins. The presentation of a mHC on the cell surface via MHCI is not guaranteed as it has to compete with a plethora of self-peptides (Griem et al., 1991). Although the immune reaction against mHC is less strong than MHC incompatibility, they should not be underestimated (de Rham and Villard, 2011).

1.4 Somatic cell nucleus transfer (SCNT)

The first experiments to transfer the nucleus of a somatic cell into enucleated oocytes resulted in the generation of Dolly. It was the first successful cloning attempt ever reported (Campbell et al., 1996). However by definition a clone is 100% genetically identical to his progenitor, which was not achieved when Dolly was born. The egg provided by the Scottish Blackface sheep (cytoplasmatic donor) whereas the nucleus was extracted from a Finn-Dorset sheep (nuclear donor). Therefore, the mtDNA was not identical to that of the donor, also postulated from numerous groups that have shown, animals derived by SCNT inherit their mitochondrial entirely or in greater parts from the oocyte and not from the donor cell (Evans et al., 1999, Hiendleder et al., 1999, Lanza et al., 2002, Byrne et al., 2007). This leads to the question if transplants derived from these clones will be recognized by the recipient immune system.

Nowadays this technique is widely used to derived murine embryonic stem cells of different genetic variations (Hochedlinger and Jaenisch, 2003). Just recently, optimized SCNT approaches allowed derivation of human SCNT derived ESCs (Tachibana et al., 2013). So far, there is no solid evidence for the immunological mechanisms inherited in the immune recognition of transplanted SCNT derived cells and it has been proposed that due to the low number of mitochondrial alleles the risk of immune response towards transplanted might be low (Drukker, 2008). In cows, cloned cells and tissues with allogeneic mtDNA could be transplanted into the nucleus donors without destruction by the immune system (Lanza et al., 2002), whereas ESCs derived via fusion of cells that carry foreign mtDNA did not form teratomas and were detected by the innate immune system (Ishikawa et al., 2010). Those data suggest an important role of mtDNA in stem cell transplant immunobiology. Thus an immune rejection due to foreign mtDNA seems possible and needs to be further investigated. The only possible solution is to use egg cells that come from the same donor of the nucleus. This would just be practical for half of the society and is therefore not ethically justifiable.

1.5 The mitochondrion

The mitochondrion is present within the cytoplasm of almost all eukaryotic cells. Along with its essential role as a generator of ATP through oxidative phosphorylation, mitochondria mediate processes such as cell division, homeostasis, steroidogenesis and apoptosis. The process of oxidative phosphorylation, which takes place within the electron transfer chain (ETC), is highly dependent on proteins encoded not only by chromosomes but also by the mitochondrial genome (St. John et al., 2010). In general, mtDNA is passed unchanged from a mother to her children, however on rare occasions parental inheritance is seen in mammalian species (Zhao et al., 2004). Increasingly the spotlight of current research turns to the role of the mitochondrial genome as defects within the mtDNA are associated with large number of clinical phenotypes (Wallace, 1999). For example; ataxia, retinitis pigmentosa and neurogenic weakness result from single point mutations in coding genes (Fryer et al., 1994). Furthermore, Encephalopathy, lactic acidosis, mitochondrial

myopathy (Goto et al., 1990) and deafness (van den Ouweland et al., 1992) are associated with mutations to tRNAs and rRNAs encoded by mtDNA.

However, immunological questions considering allogeneic mtDNA in ESC transplantation settings have so far rarely been asked (Lanza et al., 2002, Ishikawa et al., 2010).

1.5.1 mitochondrial DNA (mtDNA)

The circular, double-stranded mtDNA genome is approximately 16-17 kb in size and resides in the inner membrane of the mitochondrial compartment (Tyynismaa and Suomalainen, 2009). It encodes 13 polypeptides that are key catalytic subunits of oxidative phosphorylation complexes (OXPHOS). More precisely, there are seven subunits of NADH dehydrogenase (Complex I), one subunit of cytochrome c reductase (Complex III), three subunits of cytochrome c oxidase (Complex IV) and two subunits of the ATP synthase (Complex V) (St. John et al., 2010). The remaining OXPHOS proteins, approximately 80, and the rest of the mitochondrial proteins are encoded by nuclear genes (Anderson et al., 1981). Next to these proteins the mtDNA also encodes some of its own transcriptional and translational machinery, being 22 tRNAs and 2 rRNAs. The remains, needed for proteins synthesis, are encoded by the nucleus emphasizing the semi-autonomous nature of the mitochondrial genome. Except for the displacement loop region (D-Loop), all parts of the mtDNA genome encode in some form, therefore the mtDNA lack introns, with some coding regions even overlapping (Anderson et al., 1981).

The D-loop plays an important role in mtDNA replication and transcription. However, the maintenance of mtDNA is completely mediated by nuclear-encoded transcription and replication factors. Some proteins are involved directly in mtDNA replication and others provide nucleotides for DNA synthesis (Tyynismaa and Suomalainen, 2009).

1.5.2 Immunological antigens from the mitochondrion

MHC molecules are loaded with short peptide antigens, before they are transported to cell membrane (Murphy et al., 2008). It may be possible that as

Mitochondria get autophagocytosed, peptide fragments from the mitochondrion are loaded into the MHCI molecule.

Mitochondrial proteins are mainly encoded by the nucleus and none of the 13 known proteins encoded by the mtDNA or the RNA transcripts were known to leave the mitochondrial compartment, until cytotoxic CD8+ T-cells were postulated to recognize an amino acid change in the mtDNA-gene encoding NADH dehydrogenase subunit1 (ND1). Cells carrying a point mutation in the ND1 subunit were killed by lymphocytes (Loveland et al., 1990). The N-terminus of this mitochondrial encoded ND1 subunit is also called Mtf and is part of the non-classical I-b MHC I molecule Mta. Mta consist next to Mtf, of β2-microglobulin and M3a. The last one has a striking biochemical specificity in that it binds N-formylated peptides (Shawar et al., 1990). Interestingly, prokaryotes and mitochondria start their protein translation with N-formyl methionine leading to the assumption that mitochondrial encoded proteins might bind to M3a and are presented on the cell surface. It has been demonstrated, that the N-formylated end of the mitochondrial gene cytochrome c oxidase subunit 1 (COI) binds to M3 and is, indeed, presented by H2-M3 complex on the cell surface. Furthermore, cells that carried paternal COI were lysed by cytotoxic T-cell, whereas cells with maternal COI survived (Morse et al., 1996). Since the mother only transmits mtDNA these data elucidated COI as an mHC.

In summary, there is some evidence that proteins encoded by the mtDNA are possible mHCs and are displayed at the cell surface as they bind to MHCI molecules.

1.5.3 mtDNA replication and mitochondrial biogenesis during differentiation

Several nuclear-encoded transcription and replication factors mediate mtDNA replication (Chen and Butow, 2005). One of the key-linking proteins is proposed to be the mitochondrial transcription factor A (TFAM) (Ekstrand et al., 2004). It has a molecular weight of ~25 kDa (Parisi and Clayton, 1991) and seems to have its function in the binding, unwinding and bending of mtDNA. An overexpression in TFAM leads to an increase in both the mtDNA copy number

(Ekstrand et al., 2004) and the mitochondrial-specific polymerase γ (POLG) (Ekstrand et al., 2004, Hance et al., 2005). POLG has an essential role in the maintenance of mtDNA. It mediates the replication, repair and recombination of mtDNA (Hance et al., 2005). Although it cannot initiate the replication of mammalian chromosome, POLG can replicate mtDNA (St. John et al., 2010). Beside TFAM and POLG, the helicase, Twinkle (Spelbrink et al., 2001, Korhonen et al., 2003), and the mitochondrial single-stranded DNA-binding protein (mtSSB) play a key role in mtDNA replication (Korhonen et al., 2003) . These proteins are structural components of the mitochondrial nucleoid and have also their functions in the stabilization of mtDNA during replication (Garrido et al., 2002).

The oocyte is postulated to have the highest number of mitochondria per cell in mammalian species, as it possesses 75000-100000 (Facucho-Oliveira and St. John, 2009, St. John et al., 2010), based on the principle that there are 1-2 mtDNA copies per organelle (Shoubridge and Wai, 2007). Following fertilization of the oocyte, the number of mtDNA copies in mice remains constant up to the morula stage (Thundathil et al., 2005) or is decreased in other mammalian species (May-Panloup et al., 2005, Spikings et al., 2007). These preimplantation embryos contain low numbers of spherical and immature mitochondria that are located in the perinuclear regions of the cytoplasm (Spikings et al., 2007). Additionally, mammalian embryos express either low levels of mtDNA replication factors or none. POLG and TFAM are only briefly detected between the early stages of embryogenesis (Spikings et al., 2007). Starting at the blastocyst phase, the amount and activity of mitochondria per cell varies between the cells of trophoectoderm and the inner cell mass (ICM). Trophoectodermal cells show an up-regulation in the expression of mtDNA replication factors (May-Panloup et al., 2005, Thundathil et al., 2005, Spikings et al., 2007). The mitochondria acquire a higher membrane potential and start to differentiate into elongated organelles with swollen cristae (Stern et al., 1971, Wilding et al., 2001). These cells show a higher OXPHOS activity and ATP production (Wilding et al., 2001), which is most likely because of their differentiation into cells of the placenta and the required energy levels in that process (St. John et al., 2010). In contrast, the ICM cells continue to express

low levels POLG and TFAM (Spikings et al., 2007) and, as they show high levels of pluripotency genes, progress to become ESCs. Their mtDNA copy number is progressively reduced so that they possess 30-45 copies per cell (Facucho-Oliveira et al., 2007). Moreover, undifferentiated ESCs have a relatively undeveloped mitochondrial network with poorly developed cristae (Cho et al., 2006, Chung et al., 2007). They show low levels of ATP production and low levels of oxygen consumption (Chung et al., 2007) and an increase of glycolytic enzymes, which relates to their anaerobic metabolism (Cho et al., 2006, Chung et al., 2007). The preservation of immature mitochondria, the reduced reliance on OXPHOS enzymes and the low metabolic activity in ESCs has led to the suggestion that these mitochondrial properties are important for the preservation of self-renewal and pluripotency (Facucho-Oliveira and St. John, 2009).

Successful differentiation of ESCs requires a higher allocation of ATP (Chung et al., 2007). The efficiency of ATP production via OXPHOS exceeds that of glycolysis and thus a differentiation of the immature mitochondria of undifferentiated ESCs into a more mature and active mitochondrial network was observed in fully differentiated neurons and cardiomyocytes (St. John et al., 2005, Chung et al., 2007). They expressed high levels nuclear- and mitochondrial-encoded electronic transport chain subunits, increased their ATP production via OXPHOS (Chung et al., 2007) and showed an enriched mtDNA content per cell (Filser et al., 1997, Miller et al., 2003, Mummery, 2010).

2 Aim of the study

The goal of this study was to investigate the antigenicity of SCNT derived ESCs with allogeneic mitochondria. To analyze the immunological reactions inherited in this process we wanted to answer the following questions.

1) Are ESCs recognized and rejected by an immune competent host?
2) Do allogeneic mitochondria trigger an immunological reaction, and how potent are they?
3) Does the antigenicity, due to allogeneic mitochondria, change during differentiation?
4) Which immune cells seem to play a part in the rejection process?

3 Methodology

3.1 Cell biological Methods

All procedures involving the usage of cells were performed under sterile conditions.

3.1.1 Cell culture

3.1.1.1 Murine embryonic fibroblast (MEF)

MEF cells were purchased from Applied StemCell Inc. MEF cells are derived from CF1 mouse embryos and treated by y-irradiation to stop their further proliferation. MEF cells were seeded onto 0.1% gelatin-coated plastic flasks one day before mESC were cultured on top of MEF cells in NT-ESCs medium containing KO-DMEM (Invitrogen), 15% Knockout Serum Replacement (Invitrogen), 1% Penicillin/Streptomycin (Invitrogen), 1% L-Glutamine (Invitrogen), 1% MEM-NEEA (Invitrogen), 0.05% β-mercaptoethanol (Millipore), and 1000 U/ml leukemia inhibitory factor (LIF) (Millipore).

3.1.1.2 SCNT derived embryonic stem cells (NT-ESCs)

NT-ESCs were provided by the laboratotry of Prof. Jaenisch and sent to the TSI-Lab. NT-ESCs possessed the nucleus of a Balb/C mouse and the oocyte of a B6D2F1 (BDF1) mouse [Figure 4]. NT-ESCs were maintained on top of murine embryonic fibroblasts feeder (MEF) cells for expansion in NT-ESC medium. The NT-ESC medium was changed daily and NT-ESCs were passaged at 80-90% confluence with a split-ratio of 1:10. Accutase was used for 5-8 min at 37ºC, 5% CO_2 to detach the cells. If cells

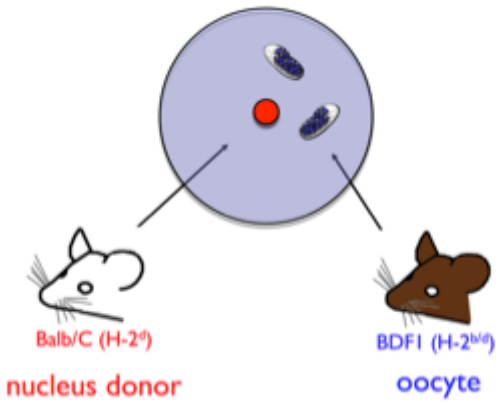

Figure 4: NT-ESCs. An enucleated unfertilized oocyte provided by a BDF1 mouse was injected with an extracted somatic cell nucleus of a Balb/C mouse.

were still attached after 8 min slight knocks against the flask were applied. The digestion was ended by the double amount of NT-ESC medium compared to Accutase used. Cells were centrifuged (800rpm, 3min) and resuspended in cell specific medium and divided according to their split-ratio.

For freezing purposes, detached mESCs were centrifuged (800rpm, 3min) and resuspended in fetal calf serum (FCS) containing 10% Dimethylsulfoxid (DMSO) and instantly stored at -80ºC using an Isopropanol-Freezing-Box allowing a steady decrease of the temperature by 1ºC/min.

Thawing was achieved through warming in a water bath at 37ºC. When the frozen cells became agile, they were quickly resuspended in NT-ESC medium and centrifuged (800rpm, 3min). The pellet was plated according to its size on either a 6 well dish, 25-T-flask or a 75-T-flask (Falcon).

For all assay condition NT-ESCs were cultured feeder free on 0.1% gelatin-coated flasks for at least 2 passages to prevent contamination with MEF cells. For feeder-free cultivation NT-ESC medium was conditioned overnight on MEF containing flasks. The conditioned medium was centrifuged (3000rpm, 3min) and transferred into a new Falcon tube to remove any cell detritus. Afterwards it was used for the maintenance of feeder-free NT-ESCs. Feeder-free NT-ESCs were passaged at 80-90% confluence using the cell dissociation buffer (Invitrogen) for 15-20 min at 37ºC, 5% CO_2. The detachment was stopped using twice the amount of NT-ESC media. Cells were centrifuged (800rpm, 3min) and divided at a ratio of 1:3-1:5.

3.1.1.3 Balb/C murine embryonic stem cells (Balb/C-mESCs)

Balb/C-mESCs were purchased from Millipore and cultivated according to the manufacturer's protocol (http://www.millipore.com/coa.nsf/a73664f9f981af8c852569b9005b4eee/31142478cf0551ad8825759100566132/$FILE/SCC052.pdf) [Status: 25.06.2013, 10:25]. Briefly, Balb/C-mESCs were thawed using a water bath at 37ºC. As the content of the vial started to move, still being frozen, cells were resuspended in Balb/C-mESC media containing DMEM (Millipore), 20% FCS-ES cell qualified (Millipore), 1% Penicillin/Streptomycin (Invitrogen), 1% L-Glutamine (Invitrogen), 1% MEM-NEEA (Invitrogen), 1% Na-Pyruvate

(Invitrogen) 2% β-mercaptoethanol (Millipore), and 1000 U/ml LIF (Millipore) and plated on top of MEF feeder in a 6-well dish. For passaging 0.05% Trypsin EDTA was used for 3min at 37ºC, 5% CO_2 to detach the cells when they reached a confluence of 80-90%. Trypsin incubation was stopped by twice the amount of Balb/C-mESC media prior to centrifugation (800 rpm, 3min) and then divided at a ratio of 1:6-1:8.

For assay condition Balb/C-mESC were cultivated similarly to feeder-free NT-ESC, using Balb/C-mESC media instead of NT-ESC media.

3.1.1.4 Murine teratoma cells

An explanted teratoma was cut into small pieces and stored in differentiation medium. The teratoma was digested using TrypLE Express (Invitrogen) for 30 min at 37ºC, 5% CO_2. Trypsin was stopped with double the amount of differentiation medium. To achieve a single cell suspension, teratoma pieces were pressed through a sterile 50µm filter and centrifuged (1200rpm, 3min). The pellet was resuspended with differentiation media and cultured in 0.1% gelatin coated cell culture flasks. Teratoma cells were passaged using TrypLE for 3min at 37ºC, 5% CO_2 with a split-ratio of 1:5 at 80-90% confluence.

3.1.2 *In-vitro* cardiomyocyte differentiation

NT-ESCs were differentiated into cardiomyocytes using the “hanging drop” method as previously described (Maltsev et al., 1994, Wang and Yang, 2008). NT-ESCs were incubated with Accutase for 5-8min at 37ºC until the cells were sloughing off the plate. Enzymatic detachment was stopped by twice the amount of NT-ESC media and gently triturated four to six times to disperse the NT-ESC with a plugged pipette. Dispersed NT-ESCs were transferred into a 15-ml Falcon tube and centrifuged (800rpm, 3min). Collected NT-ESCs were brought into a single cell suspension by up and down pipetting and plated into a T-75 flask pre-coated with 0.1% gelatin and incubated at 37ºC, 5% CO_2 for 1h. MEF feeder cells seemed to attach within this hour to the Falcon flask, whereas mESCs remained in the cultivation medium. After one hour, the medium was collected and centrifuged (1200rpm, 3min). The pellet was resuspended in fresh differentiation media (DMEM (Invitrogen) +15% FCS hi. +1% P/S + 1% MEM-

NEEA (Invitrogen) + 0.5% β-mercaptoethanol (Millipore). Cells were counted using a Neubauer-Chamber and accordingly diluted so one hanging drop contained 500 cells (500 cells per 20µl drop). The lid of a tissue culture dish was carefully inverted and rows of 20µl drops were made on the up-turned inner surface of the lid, using a multichannel pipette. The lid was again carefully inverted and put onto the tissue culture dish containing 10ml of sterile PBS (pH=7.4). After 2 days of incubation at 37ºC, 5% CO_2, 180µl fresh differentiation media was placed into a 96-well ultralow attachment plate. Hanging drops were aspirated with a multichannel pipette and transferred to the 96-well plate. Plates were put into the incubator undisturbed for the following 3 days. The day before EB transfer, 48-well tissue culture plates (Falcon) were coated with 300µl of 0.1% gelatin and stored overnight in the incubator at 37ºC, 5% CO_2,. The next day, excessive gelatin was aspirated and discarded. 300µl of fresh differentiation media was added to every well of the pre-coated 48-well plate. EBs were transferred one-by-one to 48 well plates and incubated at 37ºC, 5% CO_2. The media was changed the following day and continuously every other day to maintain the cells.

NT-ESC derived cardiomyocytes (CMs) were detached by Accutase for 5min at 37ºC, 5% CO_2 and brought into single cell suspension by continuative incubation with Collagenase IV for 15-25 min at 37ºC, 5% CO_2 until existing cell cluster were completely dissolved. Digesting of cell cluster was stopped with double the amount of differentiation media. Cells were counted as previously described.

3.1.3 *In-vivo* teratoma differentiation

In 80µl PBS (pH= 7.4), $5x10^6$ NT-ESCs were intramuscular (i.m.) injected into the right thigh of severe combined immunodeficient (SCID) beige mice. Once a tumor was palpable continuous controls of the tumor size were performed. Mice were euthanized as soon as the tumor size exceeded justifiable hindering of the animal life. General anesthesia was performed using Isoflurane. To determine the depth of anesthesia, the animal was pinched at the interspace of the toes. The anesthesia was classified to be sufficient skin when pain-mediated reflexes became absent. The skin was cut with surgical scissors on top of the sternum. After the preparation of the thorax the excision of the sternum was performed.

With clear view on the animals heart a cut aside the left and right heart margin was performed to explant the heart of the animal, resulting in the death of the animal. A cut above the right thigh was done for preparation of the tumor. After explantation, the tumor was either stored in 4% Paraformaldehyde (PFA) for histological staining or cut into pieces and stored in differentiation medium for cell culture purposes.

3.1.4 Purification of transduced cells

3.1.4.1 Puromycin killing curve

$5x10^5$ feeder-free NT-ESCs were plated into a 0.1%gelatin pre-coated 12-well plate. Puromycin was added in ascending concentration (0.1µg/ml, 0.2µg/ml, 0.4µg/ml, 0.6 µg/ml, 0.8 µg/ml, 1 µg/ml, 2 µg/ml, 4 µg/ml, 8 µg/ml) to the medium. The media were changed daily for 5 days. Cell survival was estimated using a Zeiss-Microscope.

3.1.4.2 Purification of transduced cells

Transduced NT-ESCs were cultured for at least 5 days with puromycin containing. Surviving cells were cultured in puromycin free medium for expansion and evaluation of transduction efficiency.

3.2 Animal Experiments

All procedures that involved animals were approved by the Tierschutzbehörde of Hamburg and were carried out under the Genehmigungsnummer G09/004. 6-10 week old male Balb/C, BDF1 (cross between female C57BL/6 and male DBA/2), SCID beige (B-cell, T-cell, NK-cell deficient) and Balb/C nu/nu (T cell deficient) were bought as available from The Jackson Laboratory or Charles River Inc. [Table1]. Animals were housed in cages contacting not more than 5 mice in our animal facilities with a 12:12h light-dark cycles. Immunodeficient mice were house in individual ventilated cages (IVC) to avoid any inflectional diseases. Mice had free access to standard chow and water.

Table 1. Specification of mice strains

Strain	MHCI haplotype	Deficiency	Company
Balb/C	H-2^{d}	-	Charles River / Jackson
BDF1	H-$2^{b/d}$	-	Charles River
SCID beige	H-$2^{b/d}$	T-cell, B-cell, NK-cell	Charles River
Balb/C nu/nu	H-2^{d}	T-cell	Charles River

Information obtained from: (http://www.biolegend.com/media_assets/support_resource/BioLegend_Mouse_Alloantigens.pdf) [Status: 03.07.2013, 17:30]

3.3 Immunological Methods

3.3.1 Enzyme-linked immunospot Assay (ELISPOT)

The ELISPOT assay is a common method to monitor immune responses in different species. It identifies and enumerates cytokine-producing cells at the single cell level. Each spot that develops in the assay represents a single reactive cell. Therefore it enables an insight in the cell subsets that contribute to an immune reaction against a foreign antigen.

For the quantification of the cellular immune response 6-10 week old mice were intramuscular injected with $1x10^{6}$ donor cells. Cells were injected in 60µl PBS with a 24G needle on top of a 1ml syringe. Prior to aspiration of cells, approximately 0.1-0.2ml of air was aspirated into the syringe so that no cells remained inside the needle after the injection. The day before explantation, 96 well-ELISPOT plates (Millipore) were incubated overnight with 100µl of anti-cytokine capture antibody either for murine IFNγ, IL-4 or IL-17 at a concentration of 1:200 in PBS (pH=7.2). Coated wells were blocked with Blocking solution containing RPMI 1640 +10%FCS +1%P/S +1%Glutamine for 2h at room temperature (RT) before the co-cultivation of donor and recipient cells. After 5 days, animals were anesthetized and the heart was explanted. After the death of the animal, the peritoneum was opened and the spleen was explanted cutting along the dorsal vessels. Excessive fatty tissue was removed. Spleens were harvested and carried on ice in blocking solution. To prepare cell

suspensions spleens were pressed through a 50µm nylon mesh and centrifuged at 1.200rpm for 3min. The pellet was resuspended in 1ml lysing buffer and stopped after 2min with blocking solution. After centrifugation cells were counted using a Neubauer-Chamber. Recipient cells were brought into suspension containing $1x10^7$ cells/ml in blocking solution. Simultaneously, donor cells were inhibited with Mitomycin (2mg/48ml) for 30 min at 37ºC, 5% CO_2. Donor cells were concentrated to $1x10^6$ cells/ml in cell specific media (see table). When needed, donor cells were homogenized with a 20 kHz sonicater for 3-5min. Respectively, 100µl of recipient and donor cell suspension were pipetted into pre-coated 96-well ELISPOT plate and incubated for 24h at 37ºC 5% CO_2. Afterwards, wells were washed twice with distilled water for 15 min and 5 min, respectively. After 3 further washing steps with Wash Buffer I (PBS (pH=7.4) + Tween 20 (1:2000)) wells were incubated with 100µl of the detection antibody-suspension (Dilution Buffer (PBS (pH=7.4) +2%FCS)) + anti-cytokine detection antibody (1:250)) for 2h at RT. After 3 washing steps with Wash Buffer I, 96-well plates were incubated with 100µl Horseradish peroxidase (HRP)-conjugated antibody-suspension (Dilution Buffer + HRP-conjugated antibody (1:100)) for 60 min at RT wrapped in aluminum foil. After Incubation, plates were washed 4 times with Wash Buffer I and twice with Wash Buffer II (PBS (pH=7.4)). For development 100µl of Avidin-Enzyme-Conjugate (AEC)-Substrate-Kit (1ml of Substrate+ 20µl AEC) was pipetted into the wells. Development was stopped by distilled water before the darkening of the background exceeded visibility of spots. For automated scanning and analyzing, plates were sent to Cellular Technology Limited (C.T.L., Cincinnati, Ohio, USA). After C.T.L.s quality controls, results were analyzed using Prism and SPSS.

Table 2. Cell types and correspondent cell specific media

Donor cell	Cell specific media
NT-ESC	NT-ESC media
Balb/C-mESC	Balb/C-mESC media
CMs	Differentiation media
Splenocytes	Blocking Buffer

3.3.2 Donor specific Antibodies (IgM and IgG)

Simultaneously to spleen harvesting on day 5 blood was collected for IgM donor specific antibodies (DSAs). In the case of IgG DSAs, animal sera were harvested on day 15 after injection of $1x10^6$ NT-ESCs. Following the opening of the thorax, the descending aorta was cut so that the cavity of the thorax was filled with blood. 1ml syringes were used to collect the outflowing blood and for the transfer into 1,5ml eppendorf-tubes. Tubes were centrifuged (3000rpm, 10min). Serum was aspirated and transferred into a fresh eppendorf-tube and stored at -20ºC. For DSAs, serum was decomplemented for 30min at 56ºC. After incubation serum was centrifuged (12000 RCF, 5min). 50µl serum was diluted with 100µl Fluorescence-activated Cell Sorting (FACS)-Buffer (PBS (pH= 7.4) +2% FCS) and added to 150µl SCNT-ESC cell suspension ($1x10^6$ cells/ml) in a 1,5ml eppendorf-tube. After vortexing, tubes were incubated for 45min at 4ºC. After 45min 500µl FACS-Buffer was added and tubes were centrifuged (1200rpm, 3min). The pellet was resuspended in 100µl FACS-Buffer and 10µl of anti-mouse IgM or IgG FITC conjugated antibody was added for 45min at 4ºC. After washing with 500µl FACS-Buffer and centrifugation (1200rpm, 3min) cells were resuspended in 300µl FACS-Buffer and measured with the flow cytometer.

3.3.3 Bioluminescence Imaging (BLI)

3.3.3.1 Preparation of the reporter probe D-luciferin

D-luciferin was dissolved in PBS (pH=7.4) at a concentration of 50mg/ml. For cell cultures sterile condition were achieved by filtration of D-luciferin through a sterile 0.1µm nylon mesh. Suspensions were stored at -20ºC.

3.3.3.2 Bioluminescence Imaging

Optical BLI was performed using the Xenogen in vivo imaging system and analyzed with the Living Image software. Cell cultures were screened for Luciferase after incubation with sterile D-luciferin for 5min. Dishes were put into a light-tight chamber and constantly imaged until the peek of emission of photons per second (total flux) per centimeter squared per steradian was

reached. Cells were washed once with cell specific media and continued to be cultivated.

Mice were anesthetized with Isoflurane and intraperitoneal (i.p.) injected with the reporter probe D-luciferin at a dose of 375mg per kilogram of bodyweight using a 30G Insulin-needle (BD). For imaging, the animals were placed into a light-tight chamber and repeatedly imaged by collecting the photons emitted from luciferin expressing mESCs transplanted in the animals. The exposure time for a single image changed due to the intensity of the bioluminescence emission, briefly between 10sec to 3min. Mice were measured on day 0,1 and continuously every other day until either the tumor volume enforced the euthanasia of the animal or the bioluminescence signal was absent. If the signal could not be detected, repetitive measurements on the following days were performed to verify the pre-administered results. Bioluminescence was quantified as units of photons per second (total flux) per square centimeter per steradian as previously described (Wu et al., 2003, Swijnenburg et al., 2008).

3.3.4 MHC Expression using flow cytometry

Cell types of interest (1. NT-ESC (2. +IFN-y stimulated); 3. CMs (4. +IFN-y stimulated)) were used after they had been brought into a single cell suspension. $2x10^5$ cells were resuspended in 100µl FACS-Buffer and incubated for 45min at 4ºC with 10µl PE-conjugated anti-mouse MHC-class I, class II or their respective isotype control antibody (all BD Biosciences). Cells were washed with 500µl FACS-Buffer centrifuged (1200rpm, 3min) and dissolved in 300µl FACS-Buffer for flow cytometry.

IFN-y stimulation was done by the addition of 100ng/ml murine IFN-y to the media 48h before single cell preparation and FACS-Analysis. In the case of NT-ESC IFN-y containing cell specific media was still changed daily.

3.3.5 Cytotoxic assay using LDH

The spleens of Balb/C nude mice were harvested. These mice lack the ability to form a thymus, therefore they do not have functional T- and B-cells. Spleens were carried on ice in splenocyte media (RPMI 1640 +10% FCS, +1% L-Glutamine, +1% Pencillin/Streptomycin). For single cell suspension, spleens

were handled as described above. NT-ESC (target cells) were plated in V-bottom 96-well plates (Nunc) at 10^4cells/well and mixed with splenocytes (effector cells) at different ratios (1:20, 1:40, 1:80) in cell specific media, respectively, with a final volume of 200µl. After 8h of incubation, supernatants were collected and centrifuged (1200rpm, 3min). 100µl of cell-free supernatant was placed in a flat-bottom 96-well plate. 100µl of LDH Kit–Substrate was added for 20-25min at RT, while the plate was covered with aluminum foil. For measurements of the flat-bottom 96-well plate a TECAN sunrise absorbance reader was used. The absorbance maximum was set to 492nm. Magellan 4 software was used to quantify photometric measurements.

3.3.6 NK-Cell depletion using anti-asialo GM1

3.3.6.1 Animal Treatment

Anti-asialo GM1 antibody is commonly used to degrade *in vivo* NK-cell activity (Habu et al., 1981). Anti-asialo GM1 was dissolved in 1ml PBS (pH=7.4) and stored at 4ºC. 100µl aliquots were intravenous (i.v.) or i.p injected in Balb/C and BDF1 mice. We stopped I.v. injections since the procedure was more complex and i.p. injections also showed a reduction of NKG2D+ CD3- splenocytes. Antibodies were administrated as described in the following sequence: day -4/-2/0/2/4/8/12/16/20/24/continuing. Day0 also adhered the injection of $1x10^6$ luc+-mESCs and the beginning of BLI-measurements.

3.3.6.2 Verification using NKG2D and Ly49 for flow cytometry

At day0 spleens were harvested from an untreated and antibody treated Balb/C mouse. As described above, spleens were processed and $2x10^5$ splenocytes were resuspended in 100µl FACS-Buffer and used for FACS analysis. Cells were incubated with either anti-mouse NK2D FITC conjugated and CD3 PE conjugated antibody or anti-mouse L49 FITC conjugated and anti mouse CD3 PE conjugated antibody for 45min at 4ºC. For every surface marker, 10µl of antibody was added to the cell suspension. Subsequent, cells were washed with 500µl FACS-Buffer, centrifuged (1200rpm, 3min), resuspended (300µl FACS-Buffer) and analyzed with our flow cytometer.

3.4 Staining Methods

Paraffin embedded sections of 4%PFA fixated teratomas were used for staining procedures.

For cell staining, maintenance of the cells was done on 8-well Culture Slides (BD Bioscience). Methanol was added for 10min and removed before acetone was added for 1min (methanol-acetone-fixation) to fixate the cells.

3.4.1 Histological staining Methods

Three times Xylene deparaffinization (5min each) was done before section could be rehydrated using a decreasing Ethanol row (2x 100%, 96%, 80%, 70% (first 100% ethanol step was done for 5 min, all following 3 min)). Subsequently, slides were rinsed in distilled water. After each staining procedure, sections were dehydrated using 2-3 brief dippings in 96% Ethanol and 3 x 100% Ethanol (couple of sec, 2min and 10min) before the slides were cleared with 3 x Xylene (5min each) and embedded with Vitrocloud® (for histological staining) or Prolong Gold antifade reagent (Invitrogen) (for Immunofluorescence).

3.4.1.1 Hematoxylin-Eosin (H.E.) Staining

After deparaffinization, slides were incubated in Mayer-Hämalaun for 10min followed by flowing tap water for 10min. Differentiation, extracting excessive hematoxylin from chromatin and cytoplasm, was done by a quick dip in HCl-Ethanol (0.5%HCl in 70% Ethanol) and again followed by flowing tap water for 2min. Eosin (1g Eosin in 100ml water mixed 1:1 with 1% acetic acid) was used to counterstain the cytoplasm and connective tissue (2 min). Sections were dehydrated and mounted according to the protocol written above.

3.4.1.2 Periodic Acid Schiff's (PAS)-Staining

PAS is an approved method to reveal glycoconjugates in tissue probes. Staining was performed according to the manufacturer's protocol (Carlroth-GmbH, 2011). Briefly, 1% periodic acid solution was added for 10min on deparaffinized sections and rinsed with tap water for 10min. After rinsing twice with distilled water for 2min, sections were stained with Schiff's reagent (20min, RT). Subsequently, slides were rinsed with warm tap water (35ºC minimum) for

5min and quickly rinsed in distilled water. Counterstaining of cell nuclei was performed by Mayer Hämalaun solution for 5min and additional flowing tap water for 15min. Sections were dehydrated and mounted.

3.4.2 Immunofluorescent staining Methods

3.4.2 CMs

Cells were fixated using the methanol-acetone method. After the cell fixation, plastic chambers were removed from the microscope slide. Retrieval of epitopes was achieved by heat-induction. The slides were transferred into a slide jar, containing preheated (20min, steamer) antigen retrieval solution (Dako). Slides were additionally heated for 20min in the steamer. After cooling down (RT, 45min) slides were rinsed in distilled water and sections were circled with a fat pen (Dako) to reduce the amount of antibody necessary to fully cover one section. Sections were rehydrated (2 x PBS (pH=7.4) for 3min) and blocked with Image-iT FX signal Enhancer (Invitrogen) for 30min at RT. Prior to primary antibody incubation, PBS washing (3 x 5min) was applied.

Table 3. Information of the primary antibodies used on CMs

Antibody	host	clone	subtype	concentration	company	Cata.
α-Sarcomeric actinin	mouse	monoclonal	IgG1	0.125µg/µl	Abcam	Ab9465
Connexin-43	mouse	monoclonal	IgG1	0.25µg/µl	Millipore	P17302

Primary antibodies came from the same host, making it impossible to bind a secondary antibody specific to just one primary antibody. This problem was circumvent by pre-labeling the primary antibodies with the Zenon® Kit. Solutions were calculated as shown beneath.

Table 4. Calculation of antibody solution composition according to Zenon® Kit

1st Antibody	2nd Antibody	Dilution of 1st Ab	Volume of 1st Ab	Mass of 1st Ab	Volume of 2nd Ab	Volume of Blocking Sol.
α-Sarcomeric actinin	Zenon Tricolor Alexa Fluor 633	1:50	4µl	0.5µg	2.5µl	2.5µl
Connexin-43	Zenon Tricolor Alexa Fluor 488	1:200	1µl	0.25µg	1.25µl	1.25µl

Rules: the proportion of the Mass of the 1st antibody to the Volume of the 2nd antibody should be 1:5, thus 2.5µl AF633 for α-Sarcomeric actinin and 1.25µl

AF488 for Connexin-43. Blocking solution is always equal to the secondary antibody.

To prepare 200µl antibody mixture, 4µl α-Sarcomeric actinin (1:50) and 1µl Connexin-43 (1:200) were added into two Eppendorf tubes, respectively. Secondary antibodies from the Zenon tricolor Mouse IgG1 labeling kit (Invitrogen) were added into corresponding tubes and vortexed. After a short spin, tubes were kept in the dark for 5min to enable pre-labeling. Blocking solution was added with an additional short spin and another waiting period for 5min. Subsequent, primary antibody diluent (Dako) was added to meet the required amount of antibody mixture (in this case 200µl, so 187,5µl primary antibody diluent).

With prepared antibody solutions, sections were incubated at 37ºC for 90min. PBS washing (3 x 10min) and 4% PFA post-fixation (15min) were performed before DAPI was added for 10min. Slides were rinsed with distilled water and mounted with Prolong antifade Gold (Invitrogen) and dried overnight at RT in the dark before they were imaged using the PerkinElmer UltraView Vox system connected to a Zeiss Avio Observer Z.1 microscope with 405nm/440nm/488nm/515nm/561nm/640nm laser lines, found at the UKE Microscopic Imaging Facility (umif).

3.4.3 Living cell Staining Methods

3.4.3.1 Nonyl acridine orange (NAO) staining of unfertilized murine oocytes

NAO-Staining was similarly performed as described (Keij et al., 2000). NAO was dissolved in 100% Ethanol (conc. 1mg/ml). Stock solution was stored at 4ºC and protected from light. An additional 1:1000 dilution was necessary for the NAO working solution.

Unfertilized murine oocytes were gathered from hyperovulating mice from our animal breeding facility. Oocytes were transported in PBS (pH=7.4) and washed several times. 10µl/ml (final conc. 2 µg/ml) of the NAO working solution was added and incubated in the dark for 15 min at 37ºC, 5% CO_2. Oocytes were

washed three times and subsequently 4',6-diamidino-2-phenylindole (DAPI; 1µg/ml) was applied for 10min in the dark at RT. Oocytes were mounted using Gold Anti-fade®. Since the oocytes were not fixated on a microscope slide, we transferred them with a 200µl eppendorf-pipette.

3.4.3.2 Tetramethylrhodamine methyl ester perchlorate (TMRM)

NT-ESCs, murine teratoma cells and CMs were cultured on 0.1% gelatin pre-coated confocal dishes (glass-bottom dish) in cell specific media. High centrifugation of the TMRM stock solution (13.500 rpm, 5min) was performed before the stock solution was added to cell specific media (1µl/ml) as well as Hoechst (0.01µl/ml). Incubation was done for 30min at 37ºC, 5% CO_2 followed by three washing steps with cell specific media. Observations were performed by confocal microscopy. Intensity analyses of TMRM-staining were done with the Volocity software. Intensity was measured by the mean of fluorescence emitted per voxel (≤ 0.11µm).

3.5 Physiological Characterization

3.5.1 Contraction measurement

After differentiation into cardiomyocytes, occurring beating areas were observed under a Zeiss-microscope. Beating cell clusters were filmed with a SONY DSC-W30 photo camera. Contractions were counted and measured in contractions per minute.

3.5.2 FURA-2

Fura-2 is an indicator for the measurement of Ca^{2+}-concentrations, which is excited by Ultraviolet (UV) light. Fura-2 binds to Calcium, which changes its conformation and therefore its wavelength of maximum excitement. Ratios of emission made at 340nm and 380nm correlate with the Ca^{2+}-concentration inside the region of interest (ROI) (Grynkiewicz et al., 1985, Knot et al., 2005, Laude and Simpson, 2009). Fura-2 cannot penetrate the cell membrane. To enable cell membrane passaging hydrophobic ester derivatives (e.g. Acetoxymethyl) are bind to the dye. As Fura-2-AM penetrates the cell membrane, unspecific esterases will hydrolyze the cytosolic Fura-2-AM,

imprisoning it inside the cytoplasm (Lavis et al., 2011). For CMs, cells were loaded with 10μM Fura-2-AM for 30min at 37ºC, 5% CO_2. Exposure time for 340nm was set to 40ms and for 380nm to 170ms. The time between each picture was set to 100ms. Analysis of acquired data was performed with the MetaFluor program.

3.6 Statistical analysis

Data is presented as mean plus or minus the standard deviation (SD). Unpaired Student's t-test was used to compare two unrelated groups. For a direct comparison of more then two groups a two-way ANOVA *post-hoc* Bonferroni was performed. Differences were considered significant if $p \leq 0.05$. All analyses were done in SPSS 18.0 and GraphPadPrism 4.0.

4 Materials

Antibodies for flow cytometry	Company	Clone
anti-mouse MHC class I PE conjugated	BD Bioscience	AF6-88.5.5.3
anti-mouse CD 3 FITC conjugated	BD Bioscience	17A2/145-2C11
anti-mouse IgG-antibody FITC conjugated	Sigma Aldrich	F2883
anti-mouse IgM-antibody FITC conjugated	Sigma Aldrich	F9259
anti-mouse Ly49 PE conjugated	BD Bioscience	5E6/A1/3D10/YLI-90
anti-mouse MHC class II PE conjugated	BD Bioscience	M5/114.15.2
anti-mouse NKG2D PE conjugated	BD Bioscience	CX5
Isotype Control IgG2a	BD Bioscience	G115-178
Isotype Control IgG2b	BD Bioscience	MPC-11

Cell stimulation	Company
murine IFN-y	Peprotech

Cell types	
Balb/C embryonic stem cells (Balb/C-ESC)	Millipore
Murine embryonic fibroblast (MEF)	Applied Stemcell

Animal treatment reagents	
anti-asialo GM1	Wako
D-luciferin	Biosynth

Reagents for cell culture	Company
Accutase	Stemcell
Cell dissociation buffer	Invitrogen
Collagenase IV	Gibco
Fura-2-AM	Invitrogen
Mitomycin C	Sigma Aldrich
Pluronic	Invitrogen
Trypan blue solution, 0.4%	Sigma Aldrich
TrypLE Express	Invitrogen

Reagents for Histology	Company
4',6-Diamidin-2-phenylindol (DAPI)	Invitrogen
Antigen retrieval solution	Dako
Eosin	Roth GmbH
Fat pen	Dako
Hämalaun	Böhler
Hoechst	Invitrogen
Image-iT FX signal Enhancer	Invitrogen
Nonyl acridine orange (NAO)	Invitrogen
PAS	Roth
primary antibody diluent	Dako
Prolong Gold antifade	Invitrogen

Secondary antibody diluent	Dako
Tetramethylrhodamine, methyl ester (TMRM)	Invitrogen
Vitro-Clud®	R. Langenbrinck
Zenon Tricolor Mouse IgG1 labeling Kit	Invitrogen

Antibodies for immunofluoresence	**Company**	**Clone**
alpha-Sarcomeric actinin	Abcam	EA-53
Connexin-43	Millipore	4E6.2

Media and supplements	**Company**
2-beta-mercaptoethanol 100x	Millipore
Dimethyl sulfoxid (DMSO) for cell culture	AppliChem
DMEM	Invitrogen
Dubelcco´s Phosphate Buffered Saline pH= 7.2 (PBS)	Invitrogen
Dubelcco´s Phosphate Buffered Saline pH= 7.4 (PBS)	Invitrogen
Fetal bovine serum heat inactivated (FCS)	Invitrogen
Fetal calve serum ES cell qualified (FCS-ES)	Millipore
Knockout replacement serum	Invitrogen
KO-DMEM	Invitrogen
L-Glutamine, 200mM	Invitrogen
leukaemia inhibitory factor (LIF)	Millipore
MEM non essential amino acids	Invitrogen
Na Pyruvate (100x), 100mM	Invitrogen
Penicillin/Streptomycin, 100x	Invitrogen
RPMI 1640	Invitrogen

Reagents for immunological assays	
murine IL-4 AB-set	BD Bioscience
murine IL-17 AB-set	BD Bioscience
murine IFN-y AB set	BD Bioscience
LDH quantification Kit	Promega
AEC development kit	BD Bioscience
96-well ELISPOT plates	Millipore

Equipment	**Company**
Anesthesia system	Caliper Life Sciences Inc.
Centrifuge	Eppendorf
FACSCalibur	BD Bioscience
Freezers	Liebherr
Freezing container, Nalgene Cryo 1ºC	Roth
Fridges	Liebherr
Incubator	Binder
IVIS Imaging System	Caliper Life Sciences Inc.

Microscope	Zeiss
Neubauer improved chamber	Marienfeld
Photo camera DSC-W30	Sony
Photometric reader "Sunrise"	Tecan Group Ltd.
Pipets	Eppendorf
Racks	Roth
Sonicater	Hielscher Ultrasound Technology
Steamer	Braun
Sterile bank, class II standard	Thermo Scientific
Vortex-Genie 2	Scientific Industries
Waterbath	Eppendorf, GFL

Surgical equipment

BD Insulin 0.5ml 30Gx8mm	BD
BD Microlance 24G	BD
Surgical forceps	Braun
Surgical Scissors	Braun

Software	**Company**
FlowJo8.7	Tree Star Inc.
GraphPrism	GraphPad Software, Inc.
Living Image	Caliper Life Sciences Inc.
Magellan 4	Tecan Group Ltd.
MetaFluor®	Molecular Devises
SPSS	IBM
Volocity	PerkinElmer Inc.

Consumables	**Company**
8-well CultureSlide	BD
confocal dishes	MatTrek
FACS Flow, 20L	BD
Pipette tips	Sarstedt
Sterile surgical blades	Braun
Syringe	Braun
Tissue culture flasks	Sarstedt
Tissue culture plates	Sarstedt&Greiner
V-bottom culture plates	Nunc

5 Results

5.1 Mitochondria within unfertilized oocytes

Ms. R. Reusch from the animal facility at the University-Hospital Hamburg-Eppendorf provided us with unfertilized oocytes. We identified mitochondria in unfertilized mature oocytes with a NAO staining and the nucleus with a DAPI staining [Figure 5]. Mitochondrial staining by NAO is attributed to the binding of NAO to cardiolipin in the inner mitochondrial membrane (Petit et al., 1992). This data highlights that unfertilized oocytes carry numerous amounts of mitochondria.

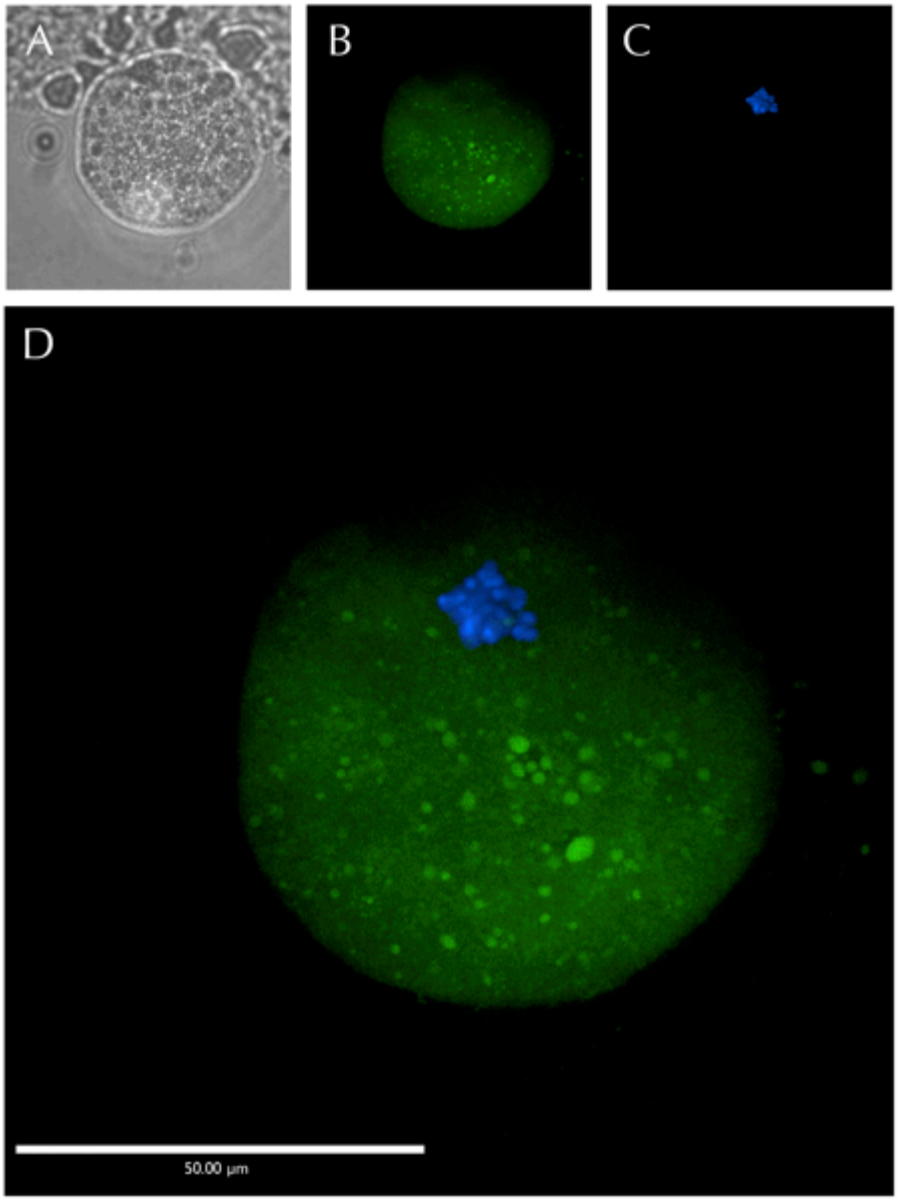

Figure 5. Verification of mitochondria inside an oocyte: A) Bright field image; B) Oocyte is stained with NAO. Mitochondria are stained as NAO binds to cardiolipin in the inner mitochondrial membrane; C) Oocyte nucleus is stained by DAPI; D) merged (magnification: 600x)

5.2 Characterization of NT-ESCs

We cultured NT-ESCs under typical mESC culture conditions, namely the cultivation on inactivated MEFs under the presence of LIF. Under these conditions NT-ESC showed typical pluripotent stem cell morphology and expressed key pluripotent genes such as OCT4, Sox2, SSEA1 and AP. The ability of self-renewal and pluripotency of ESCs is granted for unlimited generations. We obtained mESCs for more than 50 passages without signs of differentiation. When cultured under feeder-free conditions cells started to differentiate within the first feeder-free passage. Passaging feeder-free cells 2-3 times with cell dissociation buffer eliminated differentiate cells

To confirm the pluripotency of NT-ESCs *in vivo*, $5x10^6$ and $1x10^6$ NT-ESCs were injected i.m. into the right thigh of SCID beige mice. Mice that received $5x10^6$ NT-ESCs formed visible teratomas after 3 weeks of injection [Figure 6].

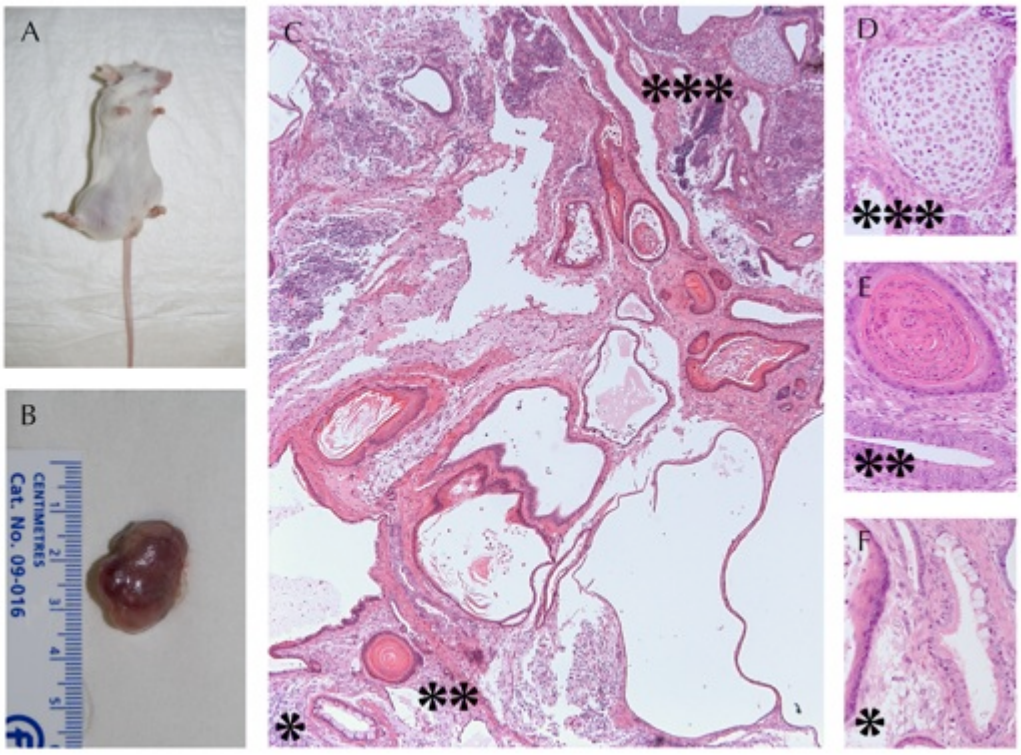

Figure 6. Pluripotency verification of NT-ESCs by teratoma formation *in vivo*: A) $5x10^6$ NT-ESC formed a visible tumor in SCID beige after 3 weeks; B) explanted teratoma; C) H.E staining of a teratoma section (magnification: 25x); D) mesodermal lineage is represented by cartilage; E) representative image of epidermis indicating ectodermal cell lineage; F) endodermal derived cell types are identified by bronchial epithelium (magnification D-F: 400x). Pathologist Dr. J. Velden performed identification of germ layer specific cell types.

Animals, injected with $1x10^6$, showed tumor growth after 9 weeks. The microscopic analysis of H.E. and PAS stained sections of the explanted teratoma revealed tissue types of all three germ layers within the teratoma, including cartilage, epidermis, bronchial epithelium, brain and skeletal muscle [Figure 6]. In summary, NT-ESCs exhibited characteristics of pluripotent embryonic stem cells, allowing them to differentiate into any cell type within the mammalian body.

5.3 Cardiac differentiation of mESC *in vitro*

After cardiogenesis of ESC in EB cultures, we observed spontaneously contracting cell clusters within the first 7 days after EBs were plated on overnight 0.1% gelatin pre-coated 48-wells plates or confocal dishes. Beating areas could be maintained for up to 21 days, before either the cells started to detach or the cell number outgrew the possible cultivation size of the plates.

CMs showed recurring contraction at a rate of 62.95±9.28 contractions/minute (n=7). To visualize calcium changes inside CMs Fura-2-AM measurements were performed at the Laboratory of PD Dr. R. Kiefmann. Fura-2-AM measurements showed changes in the ratio of 340/380nm before and during contraction in beating cell cluster [Figure 7]. This experiment was only performed once, therefore these results are rather descriptive.

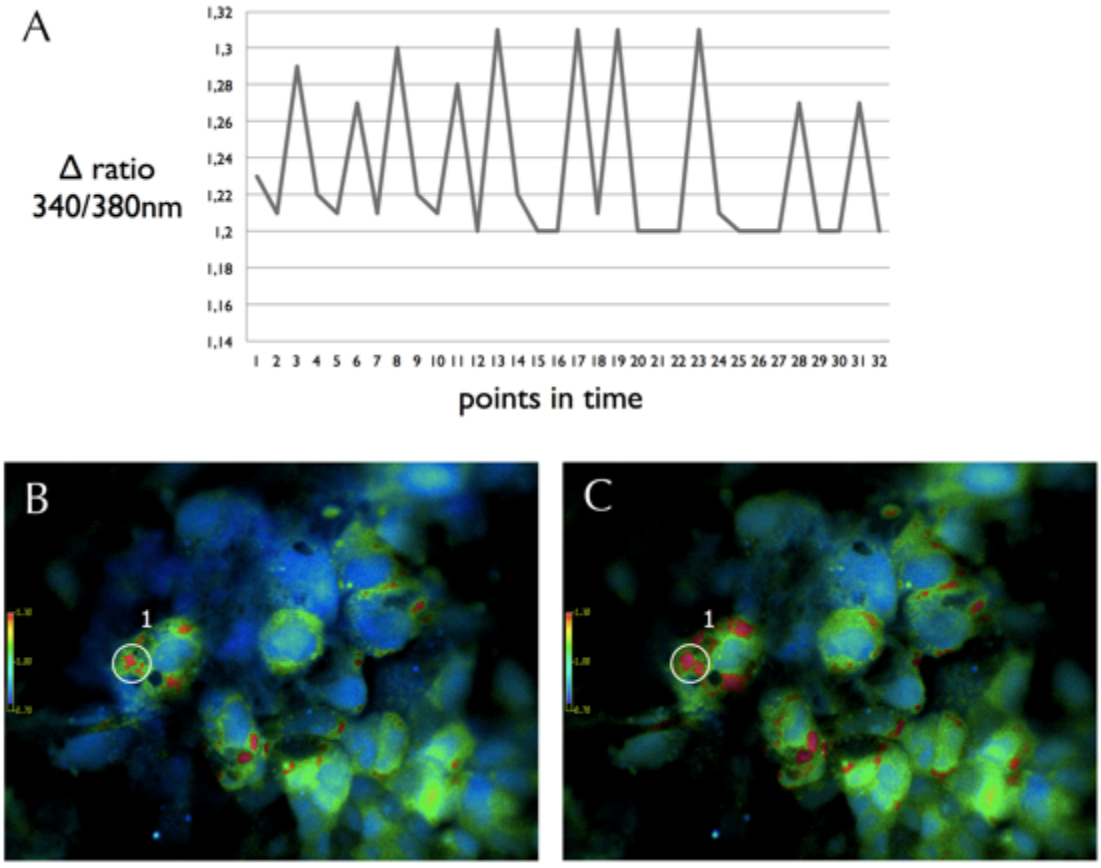

Figure 7. Fura-2AM measurements of CMs: Fura-2 enables Ca2+ induced fluorescent measurements, when a ratio is made between the emission at 340 and 380nm. A) Data shown represents 340/380nm ratio measurements at sequent points in time of the ROI, seen in B and C. Between every point in time 100ms elapsed. B) Snapshot before contraction, C) Snapshot during contraction.

Furthermore, CMs expressed cardiac markers such as α-Sarcomeric actinin and Connexin 43 [Figure 8]. These findings, in addition to spontaneously recurring contractions visible under the light microscope imply cardiac alike muscle cells.

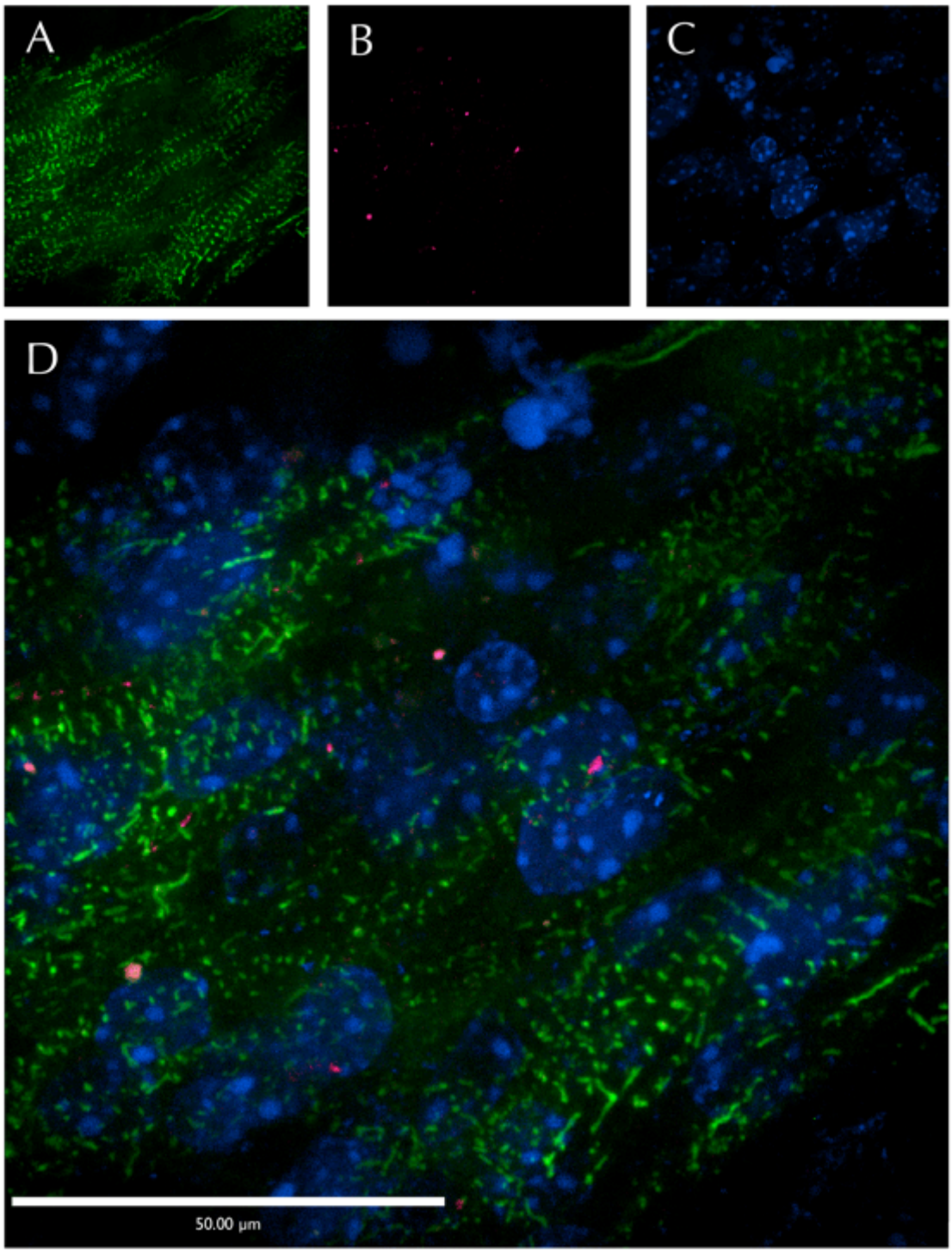

Figure 8. Verification of cardiac muscle specific structures: CMs were stained with cardiac muscle specific antibodies and visualized using confocal microscopy. A) α-Sarcomeric actinin (green); B) Connexin 43 (purple); C) Cell nuclei are stained with DAPI (blue); D) merged (magnification: 600x)

5.4 Mitochondrial differences

The amount of mitochondria within cells differs in relation to cell function. There is data suggesting that the amount of mitochondria can be evaluated indirectly through the level of activation (Filser et al., 1997, Miller et al., 2003, Mummery, 2010). Mitochondrial activation can be measured by TMRM. Accumulation of TMRM results in a red shift in wavelength and acts as a fluorescent probe to monitor the membrane potential of mitochondria ($\Delta\psi$). If $\Delta\psi$ increases so does the fluorescence intensity (Chazotte, 2011). TMRM measurements showed differences in the $\Delta\psi$ of NT-ESC, murine teratoma cells and CMs.

Our findings revealed that ESCs (TMRM-intensity: 1628.35±174.6, n=6) emit a significantly lower amount of fluorescence intensity compared to murine teratoma cells (TMRM-Intensity: 1628.35±174.6, n=3 ($p=0.026$) and CMs (TMRM-Intensity: 5262.051±992.97, n=5 ($p<0.001$)). Murine teratoma cells also emitted significantly less fluorescent dye than CMs ($p = 0.004$) [Figure 9]. Numbers of samples varied because we could only find a limited amount of representative areas.

We identified CMs by their circadian contractions. They showed the highest fluorescence intensity, implying that they contain cells with the most activated mitochondria and indirectly with the most mitochondria per cell.

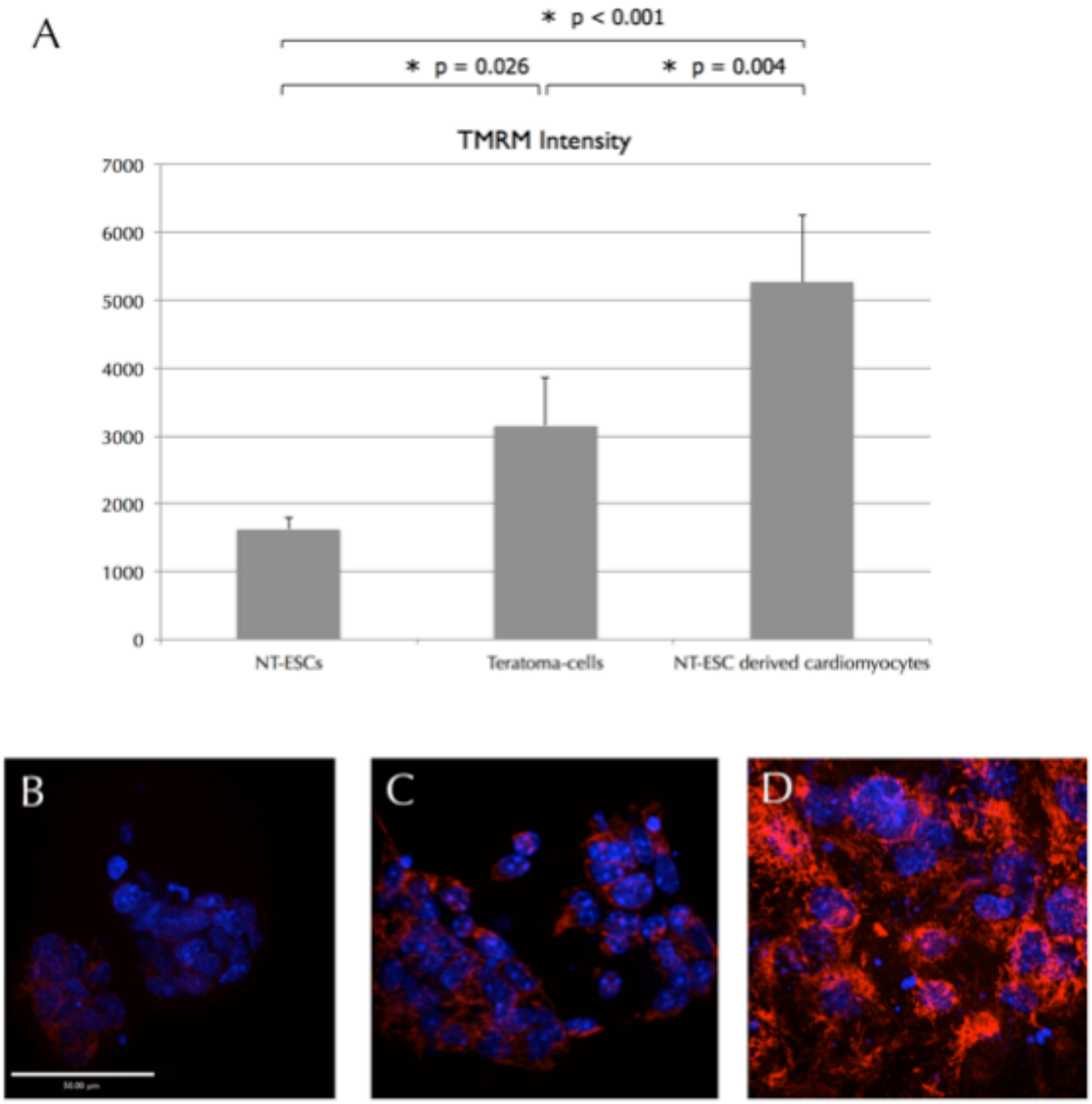

Figure 9. Increasing mitochondrial membrane potential (∆ψ) with differentiation: NT-ESC, Teratoma-cells and CMs were stained with TMRM and visualized using confocal microscopy. TMRM-Intensities were quantified with Volocity software. A) Mean values of fluorescent dye of NT-ESCs, Teratoma-cells and CMs. B-D) confocal images of NT-ESC, Teratoma-cells and CMs. Cell nuclei are stained with Hoechst (blue), whereas mitochondria are visible through TMRM (red) (magnification: 600x)

5.5 MHC class I and II surface marker expression

Like other groups (Drukker et al., 2002), we found low expression of MHCI-molecules, compared to their specific isotype control, on NT-ESCs (3.2±1.3 fold; n=12) and CMs (3.1±0.5 fold ; n=9) and no expression of MHCII on NT-ESC (1 fold, n=4) [Figure 10]. MHCI-level significantly increased after IFNγ stimulation for 48 hours (NT-ESC: 7.3±1.7 fold ($p<0.001$), n=5; CMs: 30±13.4 ($p=0.021$), n=4). We did not analyze MHCII levels after IFNγ stimulation for NT-

ESCs and CMs, because MHCII induction due to IFNγ stimulation has not been seen (Drukker et al., 2002). Only differentiation into hematopoietic and DC lineages appears to increase MHCII molecules (Slukvin et al., 2006, Senju et al., 2007). In summary, with differentiation and in an inflammatory milieu MHCI expression of ESCs rises.

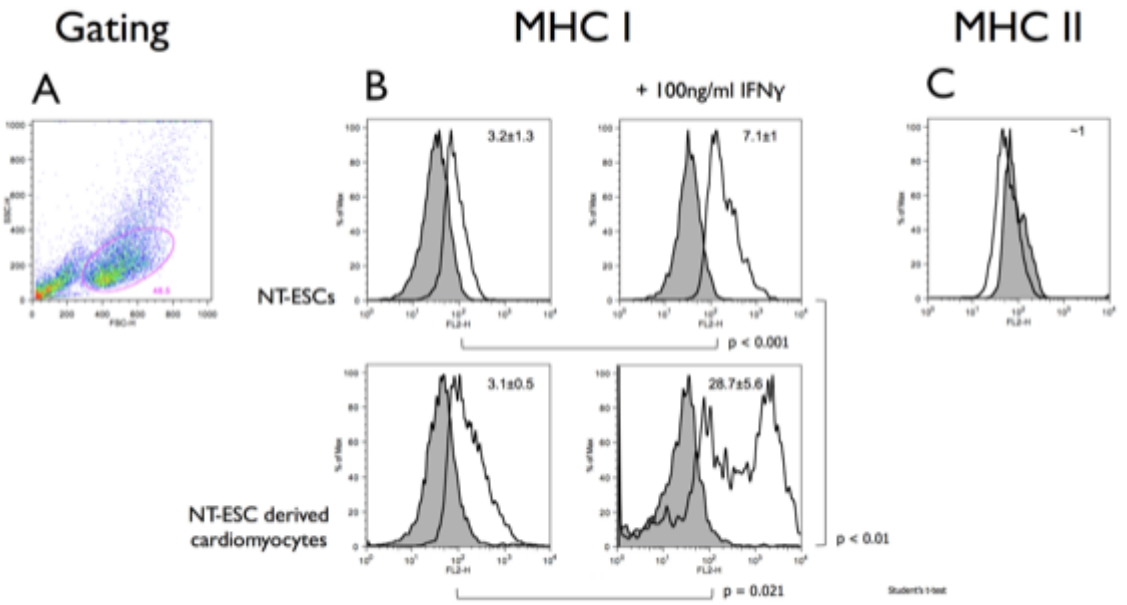

Figure 10. MHC-class I and II expression on NT-ESCs and CMs. Cells were stained with PE conjugated anti-MHCI and anti-MHCII antibodies. Isotype controls (grey) were PE conjugated anti-IgG2a and -IgG2b, respectively, as advised in the manufacture's protocol. A) Characteristically gating for NT-ESCs; B) Representative flow cytometry histograms (*y-axis*) and graphical demonstrations (*x-axis*) of MHCI expression on the cell surface of either NT-ESCs (n=12) or CMs (n=9) compared to IFNγ-stimulation (NT-ESC (n=5); CMs: (n=4); C) NT-ESC's MHC-II level (n=4). Student's t-test was performed for statistical analysis.

5.6 Immunological investigations

ELISPOT, DSAs and BLI assays explored involvement of the adaptive immune system in the rejection of NT-ESC with allogeneic mtDNA. To investigate further subsets of the immune system, we performed a cytotoxicity assay and BLI measurements of either anti-asialo GM1 treated animals or Balb/C_nu/nu.

5.6.1 ELISPOT

5.6.1.1 Direct allorecognition

For ELISPOT assays, and throughout the study, groups differed in either their mitochondria- (NT-ESC in Balb/C mice) or nuclei-incompatibility (NT-ESC in BDF1 mice) of transplanted cells to the host genetic background.

Immunodeficient SCID beige mice were the negative control. BDF1 mice received Balb/C-mESC (mitochondria and nuclei are foreign) for the positive control [Figure 11]. We analyzed IFNγ, IL-4 and IL-17 cytokine production as Th-1 cells mainly produce IFNγ, whereas IL-4 is characteristic for Th-2 cells and IL-17 is the hallmark cytokine of Th-17 cells.

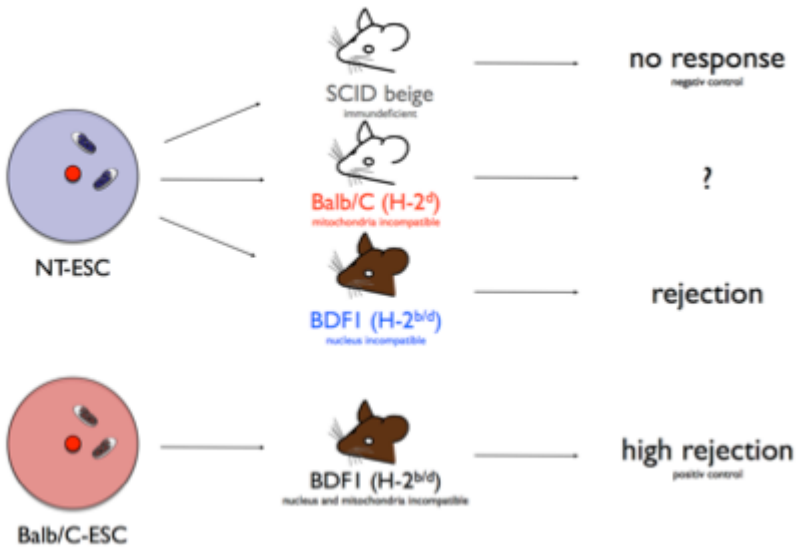

Figure 11. Animal groups. NT-ESCs were injected into SCID beige (negative control), Balb/C (mitochondria incompatible) and BDF1 mice (nucleus incompatible). Balb/C-ESCs were injected into BDF1 for positive control, since both mitochondria and the nucleus carry allogeneic antigens. Assumed outcomes are noted on the right hand side.

Five days after transplantation, splenocytes of the mitochondria and nuclei-incompatible groups secreted significantly higher amounts of IFNγ (mitochondria-incompatible: 153.8±70.7; nucleus-incompatible: 128.8±60.9; n=10), compared to SCID beige mice (IFNγ: 25.44±15.61; $p<0.001$; n=6) [Figure 12]. Cytokine-release of IL-4 seemed increased, but was not significant to the negative control (mitochondria-incompatible: 49±58.9, n=10; nucleus-incompatible: 54.8±40.7, p=ns n=10; SCID beige: 24.39±12.67; n=6). IL-17 production was not significantly elevated for the mitochondria- and nucleus-incompatible group in comparison to the negative control (mitochondria-incompatible: 7.2±7, n=10; nucleus-incompatible: 3.1±3.7, n=10; SCID beige: 4.5±6.2; n=6). Counted IL-17 Spots were low compared to other cytokines, so conclusions about the Th-17 activation have to be evaluated critically. ELISPOT analyses adhere to this limitation, being a single cell assay. IFNγ, IL-4 and IL-17 production did not significantly differ between the mitochondria- and nucleus-incompatible groups (p=ns). For all cytokines both mitochondria- and nuclei-

incompatible animals were significantly lower than the positive control (IFNγ: 655.1±89.1; IL-4: 147.9±33.7; IL-17: 14.7±6.6; p<0.001; n=6) [Figure 12]. These data suggest a cellular immune activation towards foreign mitochondria and nuclei.

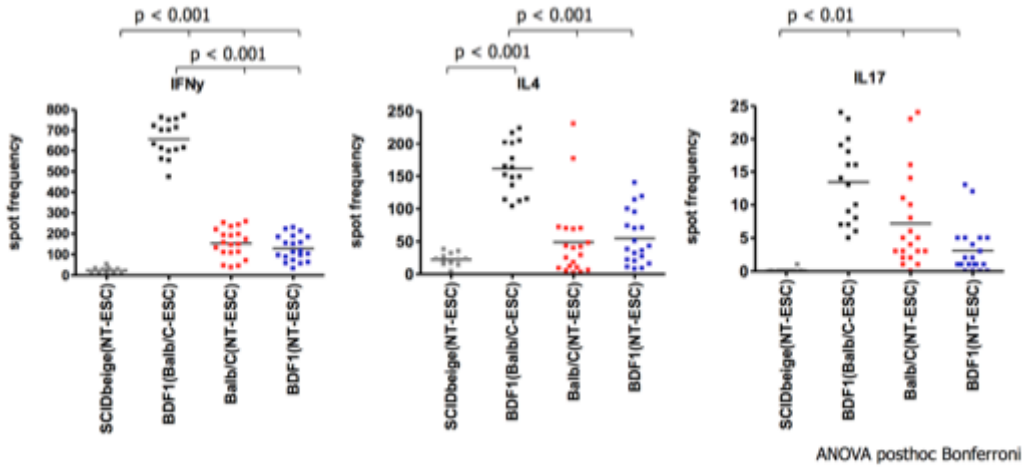

Figure 12. Direct allorecognition ELISPOTS. 1x10^6 NT-ESCs or Balb/C-ESCs were injected into SCID beige, Balb/C and BDF1 mice, as described above. Systemic immune response was investigated after 5d with unidirectional ELISPOT assays. Spots were automatically enumerated using an ELISPOT plate reader (CTL). Two-way ANOVA *posthoc* Bonferroni was performed for statistical analysis.

5.6.1.2 Indirect allorecognition

Besides direct allorecognition, we investigated an indirect allorecognition by sonication of stimulatory cells for ELISPOT analyses. Sonication disrupts cell membranes and releases cellular components. For indirect allorecognition antigen presenting cell (APC), such as macrophages, dendritic cell and B-Cells, need to take up these antigen, process it and present it to nearby immune cells. Roughly 40% of murine splenocytes have been linked to the APC population (Pinchuk and Filipov, 2008). Stimulation of splenocytes with sonicated NT-ESC showed a significant IFNγ production for the mitochondria-incompatible group (IFNγ: 61.2±54.2, p=0.03 n=10) but not for the nuclei-incompatible group (IFNγ: 20.5±19.4, p=ns, n=10) when compared to SCID beige mice (2.2±3.7, n=4). The positive control was again highly significant to all groups (IFNγ: 148.3±73.1, p<0.001, n=6). IL-17 and IL-4 production were absent or not significant, compared to the negative control (mitochondria: IL-4: 25.45±25.63, IL-17: 4.7±7.3, n=10; nuclei: IL-4 2.1±3.9, IL-17: 2.1±3.9, n=10; SCID beige: IL-4: 2.2±3.7, IL-17: 0.6±1.2, n=4) [Figure 13]. These data suggest that foreign

mitochondrial proteins are assimilated, processed and presented to close immune cells.

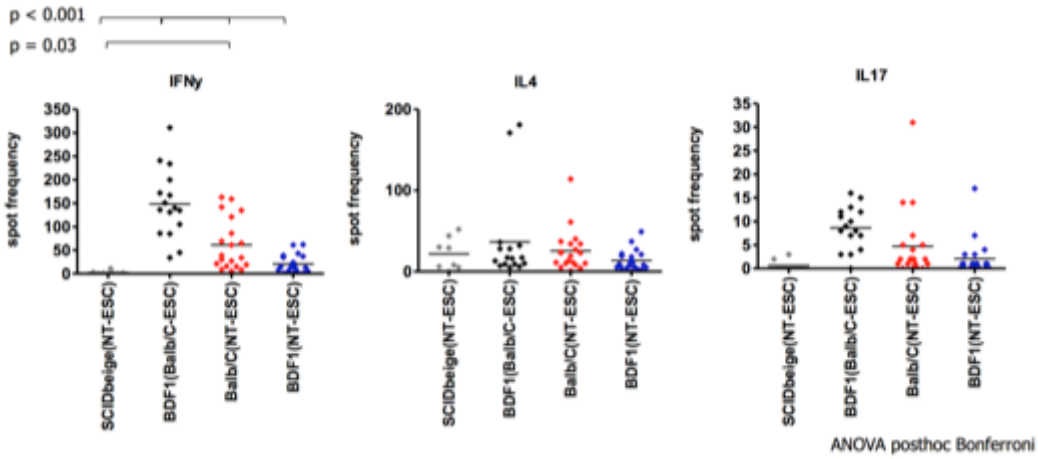

Figure 13. Indirect allorecognition ELISPOTS. Next to direct allorecognition stimulatory cells were sonicated, destructing cellular structures. APCs had 24h to process and present ESC's antigens to lymphocytes *in vitro*. Spots were automatically enumerated using an ELISPOT plate reader (CTL). Two-way ANOVA *posthoc* Bonferroni was performed for statistical analysis.

5.6.1.3 Re-injection for adaptive immune response

Previously described ELISPOT analyses implied a cellular mediated immune recognition of foreign mitochondria, therefore we hypothesized that a re-stimulation of T-cells due to a re-injection with foreign antigens would enhance the immune response of recipient mice. To demonstrate an adaptive immune response towards foreign mitochondria we re-injected $1x10^6$ NT-ESC into the contralateral thigh of mitochondria- and nuclei-incompatible mice (n=6) 3 days after the first injection. After 5 days of the first transplantation we performed ELISPOT assays. Re-injected animals of the mitochondria-incompatible group showed a significantly higher cytokine production for IFNγ, IL-4 and IL-17 in comparison to singular injected animals (IFNγ: 356±101.2, p<0.001; IL-4: 113.5±76.9, p=0.013; IL-17: 99.1±15.8, p=0.014). The nucleus-incompatible group was significantly higher for IFNγ and IL-4 but not for IL-17 (IFNγ: 384.4±224.3, p<0.001; IL-4: 92.8±64.3, p=0.02; IL-17: 4.1±2.8, p=ns) [Figure 14]. In conclusion, re-stimulation results in a greater immune response towards foreign mitochondria and nucleus, which possibly involves the adaptive immune system.

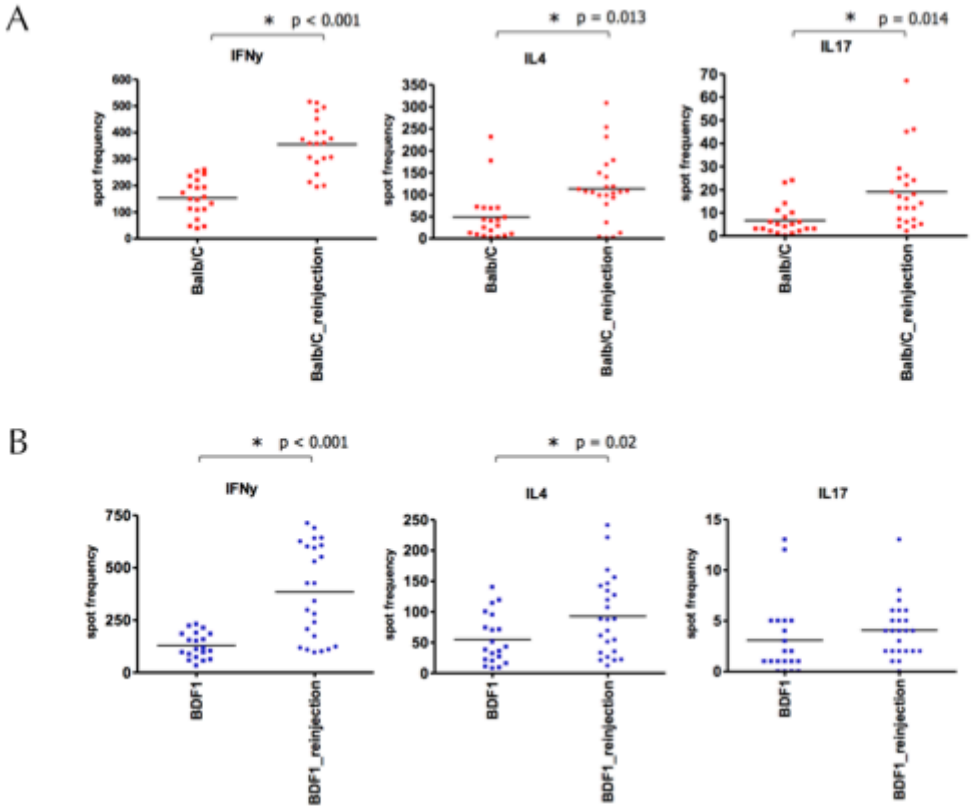

Figure 14. Re-injection ELISPOTs for adaptive immune response (3d+2d). 1x10^6 NT-ESCs were injected into Balb/C and BDF1 mice. After 3d animals received a re-injection with 1x10^6 NT-ESCs in the contralateral thigh. Adaptive immune response was investigated after additional 2d with unidirectional ELISPOT assays for IFNγ, IL-4 and IL-17. A) Mitochondrial-incompatible re-injection animals compared to singular injected animals. B) Results for the nucleus-incompatible mice. Spots were automatically enumerated using an ELISPOT plate reader (CTL). Student's t-test was performed for statistical analysis.

5.6.2 *In vivo* tracking of transplanted NT-ESCs using Bioluminescence imaging

NT-ESCs were stably transduced with a gene construct carrying firefly luciferase (fLuc) attached to a puromycin resistance gene (?) under control of a cytomegalovirus (CMV) promoter [Figure 15]. Hence, stably transduced ESCs can resist the addition of Puromycin to cell media, enabling us to purify the cell culture. Once cells are transduced they exhibit a robust correlation between fLuc expression and cell number, as shown

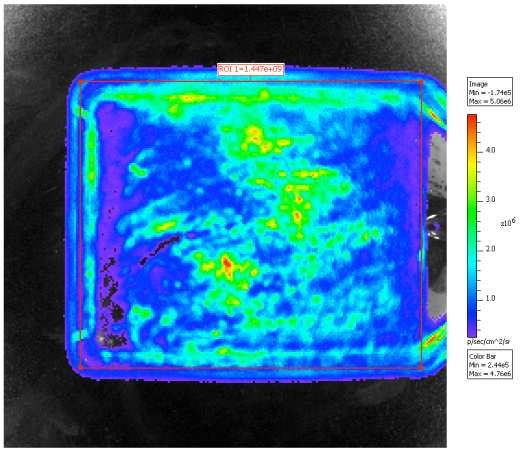

Figure 15. fLuc positive NT-ESCs. NT-ESCs were stably transduced with a gene construct encoding for fLuc driven by a CMV promotor. BLI of a T75-flask with a fLuc activity of 1.447x10^9 p/sec/cm^2/sr.

by numerous groups (Cao et al., 2006, Swijnenburg et al., 2008, de Almeida et al., 2011), which allows *in vivo* tracking of cells.

BLI measurements revealed complete rejection of NT-ESCs in mice that received mitochondria-incompatible ESCs [Figure 16]. Interestingly, 2/7 animals rejected the cells at a rather late stage (mean 53d±5.66), whereas 5/7 mice rejected NT-ESCs 5 weeks earlier (mean: 18.2d±3.35). Late-rejecting mice had palpable but not visible nodules, which were very hard in consistence. Over time they decreased in size until they completely disappeared.

In the nucleus-incompatible group 3/7 mice showed complete rejection of transplanted cells (mean: 13.67d±1.15). Interestingly, 4/7 mice did not reject transplanted cells leading to a tumor growth [Figure 17].

All immunodeficient SCID beige mice showed no signs of rejection (n=3). Even though the mtDNA only contributes to 13 proteins these data tend to give them a stronger antigenicity when compared to nuclear DNA if the immune rejection is unlikely to occur due to differing MHC haplotypes.

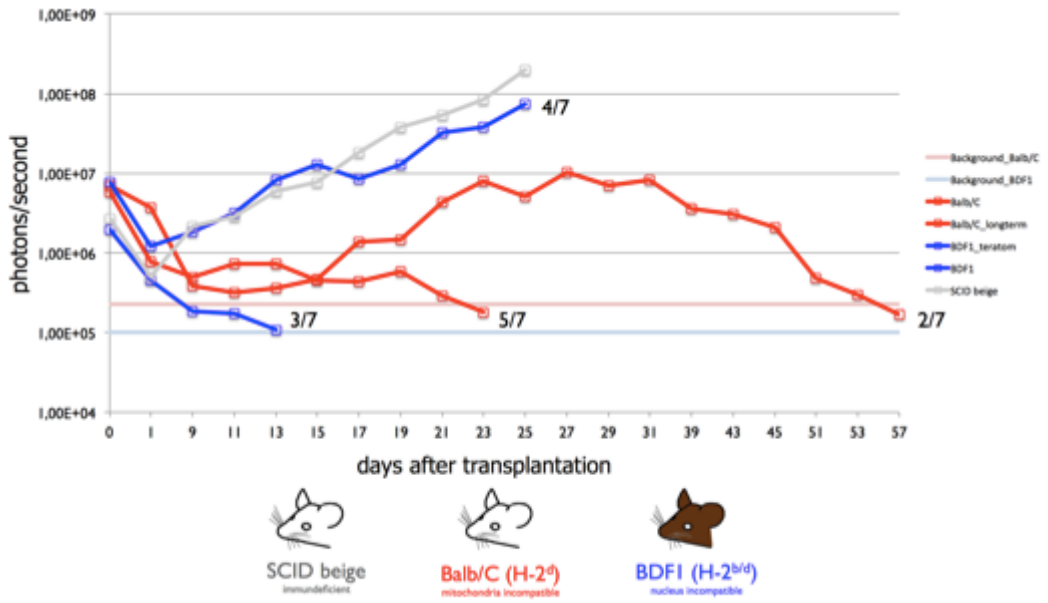

Figure 16. Cell survival *in vivo* using BLI. Balb/C, BDF1 and SCID beige mice were i.m. injected with $5x10^6$ fLuc+ NT-ESCs. Cell survival was monitored on day 0, 1, and continued every other day until the tumor growth exceeded ethical approval or no living cell could be detected. For better visibility of the graph, measurements between day 3-7 are not shown, as well as their SD. Graph represents cell survival *in-vivo* by logarithmic photon emission per square centimeter per steradian (*y-axis*) against days of imaging (*x-axis*).

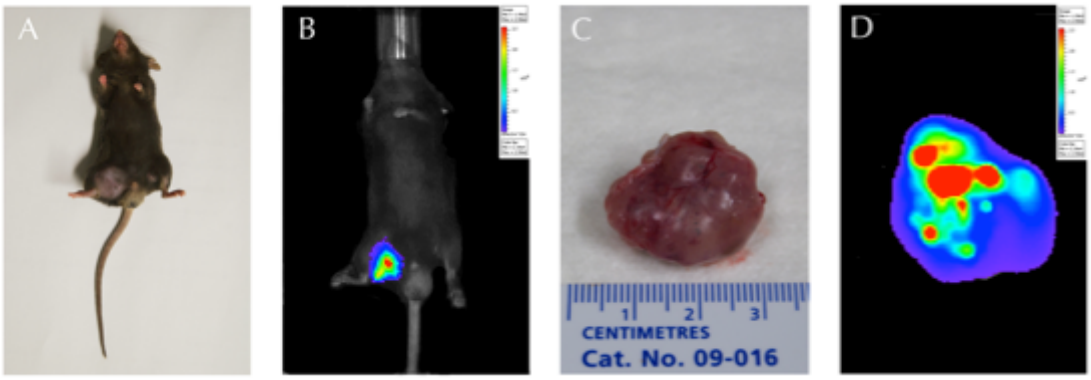

Figure 17. Tumor in nucleus-incompatible BDF1 mice. BDF1 mice were injected with $5x10^6$ NT-ESCs. After 25d animals that develop a tumor had to be euthanized as the tumor growth exceeded justifiable hindering of the animals life. A) anesthetized BDF1 mice; B) BLI image; C) explanted tumor; D) screening of fLuc positivity of explanted tumor.

5.6.3 Donor specific antibodies

5.6.3.1 IgM-antibodies

As seen in ELISPOT analyses IL-4 production of recipient mice for direct and indirect allorecognition was insignificant compared to immune deficient mice. Therefore, we did not expect to find significantly elevated IgM-levels in animal sera.

Next to the harvesting of spleens for ELISPOT analyses, we collected animal sera to allow direct comparisons between ELISPOT results and DSA production.

IgM levels were measured as mean fluorescent intensity (MFI). The mitochondria-incompatible group showed a significantly higher presence of circulating IgM-antibodies against NT-ESCs, compared to immune deficient SCID beige mice (MFI: mitochondria-incompatible 56.66±15.82; SCID beige: 4.55±1.54, n=6, $p<0.001$). Nuclei-incompatible animals showed by trend elevated IgM-levels, though these were not significantly increased (MFI: nucleus-incompatible 32.88±6.06, n=10, p=ns) [Figure 18]. The positive control (MFI: 82.23±39.59, n=6, $p<0.001$) showed a significant increase in IgM-levels compared to the nucleus-incompatible mice and the negative control ($p<0.001$). This was not observed in comparison to the mitochondrial-incompatible group (p=ns).

Furthermore we tested the sera of re-injected animals (3+2d) for circulating IgM-antibodies. Because of increased IL-4 secretion observed in ELISPOT results we expected elevated IgM levels in comparison to singular injected mice, However, only the nucleus-incompatible group showed a higher presence of IgM-antibodies against NT-ESCs (MFI: 59.39±14.58, n=6, p=0.08). IgM-levels of the mitochondria-incompatible group were insignificant (MFI: 60.72±12.51, n=6, p=ns) [Figure 18].

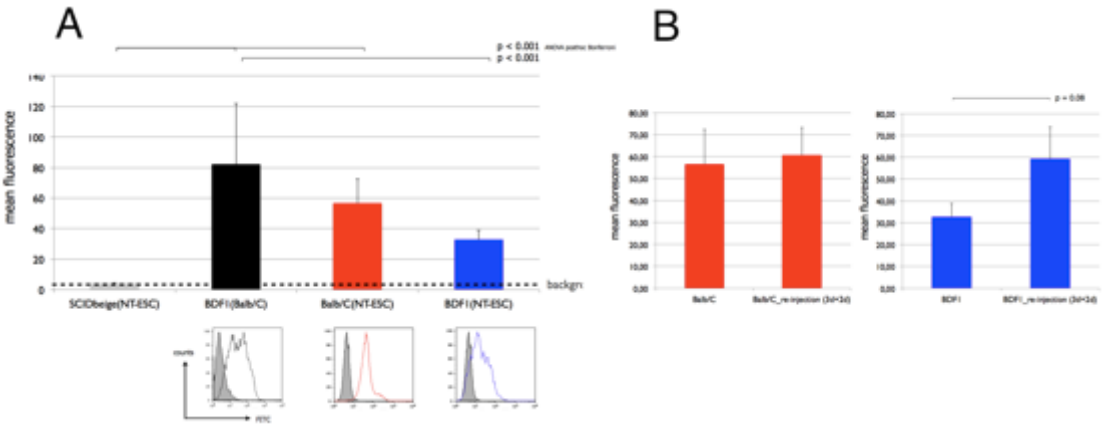

Figure 18. Humoral immune response after 5d in singular or re-injected animals. Simultaneously to spleen harvesting for ELISPOT assays animal sera was collected. Flow cytometry analysis revealed circulating donor specific IgM-antibodies in animal sera. MFI is shown for each group. A) MFI of SCID beige, allogeneic, mitochondria- and nucleus-incompatible groups, representative flow cytometry histograms (*y-axis*) and graphical demonstrations (*x-axis*) of IgM-antibody bound to donor specific cell surfaces. Statistics were achieved by two-way ANOVA *posthoc* Bonferroni B) Presence of IgM-antibodies in re-injected compared to singular injected mice for mitochondrial- and nuclear-incompatible mice. Student's t-test was performed.

Nevertheless, these data indicate an activation of the humoral immunity against mitochondrial and nuclear antigens of transplanted ESCs.

5.6.3.2 IgG-antibodies

IgG-antibody production requires an immunoglobulin class switch from IgM to IgG. A class switch leaves the antigen specificity unaffected but enables interactions with different effector molecules. Since ELISPOT analysis showed Th-1 and Th-2 cell responses we hypothesized a possible class switch from IgM to IgG. IL-4 and IFN-y trigger an Immunoglobulin class switch from IgM to IgG1 and IgG2a in mouse, respectively (Murphy et al., 2008). After 15 days of transplantation recipient animal sera showed circulating IgG-antibodies, reacting against NT-ESCs. Immunodeficient SCID beige mice acted as the

negative control (MFI of SCID beige: 9.5±0.30, n=4; mitochondria-incompatible: 132.2±25.94, p<0.006, n=6; nucleus-incompatible: 90.93±14.79, p<0.003, n=6) [Figure 19]. In our study we used the detection antibody from Sigma Aldrich, which detects IgG1, IgG2a/b and IgG3 antibodies, as mentioned in the manufacture's datasheet (http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Datasheet/7/f2883dat.Par.0001.File.tmp/f2883dat.pdf) [Status: 25.06.2013, 10:30]. Hence, a definite conclusion which IgG subclass was present cannot be made.

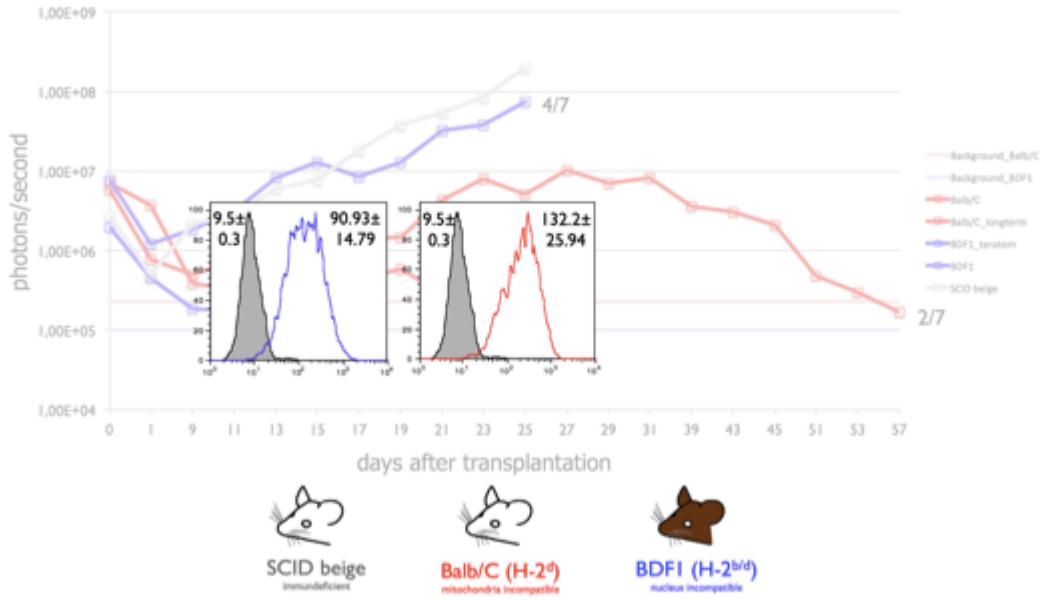

Figure 19. Circulating IgG-Antibodies. Sera were harvested after 15 days. Flow cytometry analysis revealed circulating donor specific IgG-antibodies in animal sera. Representative flow cytometry histograms are shown with corresponding mean fluorescence intensities (MFI). BLI-Curves are shown to represent the status of the animals at the point in time of serum harvesting.

5.6.4 Cytotoxic assay

Balb/C nu/nu lack a thymus and hence exhibit almost no T-cell functions despite some Th-1 positive cells. They mainly rely on T-cell independent pathways to recognize allogeneic antigens. Cell lyses due to Balb/C_nu/nu splenocytes will unlikely occur because of conventional mature T lymphocytes. If cells are lysed, Lactate-dehydrogenase (LDH) is released from the inside of the destructed cell. We measured LDH levels for our cytotoxicity assay.

LDH levels were significantly elevated in all E/T ratios, when K562 were co-cultivated with Balb/C_nu/nu splenocytes, compared to merely Balb/C_nu/nu splenocytes (Absorbance: E/T_80: K562 1.26±0.11; splenocytes 0.73±0.06, p<0.001; E/T_40: K562 1.18±0.14; splenocytes 0.48±0.03, p<0.001; E/T_20: K562 0.99±0.09; splenocytes 0.46±0.08, p<0.001; n=6). Co-cultivation of NT-ESCs with Balb/C_nu/nu splenocytes only showed at the E/T_40 ratio a significant increase of LDH levels compared to only Balb/C_nu/nu splenocytes. (Absorbance: E/T_80: mESC 0.78±0.07, splenocytes 0.73±0.06, p=ns; E/T_40: NT-ESC 0.63±0.03; splenocytes 0.48±0.03, p=0.026; E/T_20: mESC 0.48±0.01; splenocytes 0.46±0.08, p=ns; n=6) [Figure 20]. In our assay we saw an increase in LDH levels when K562 cells were incubated with Balb/C_nu/nu splenocytes in comparison to K562 cells without any effector cells (Absorbance: K562+Balb/C_nu/nu splenocytes: 1.26±0.05; K562: 0.83±0.06 p=0.003; n=6). Therefore, Balb/C_nu/nu splenocytes seemed capable to lyse human K562.

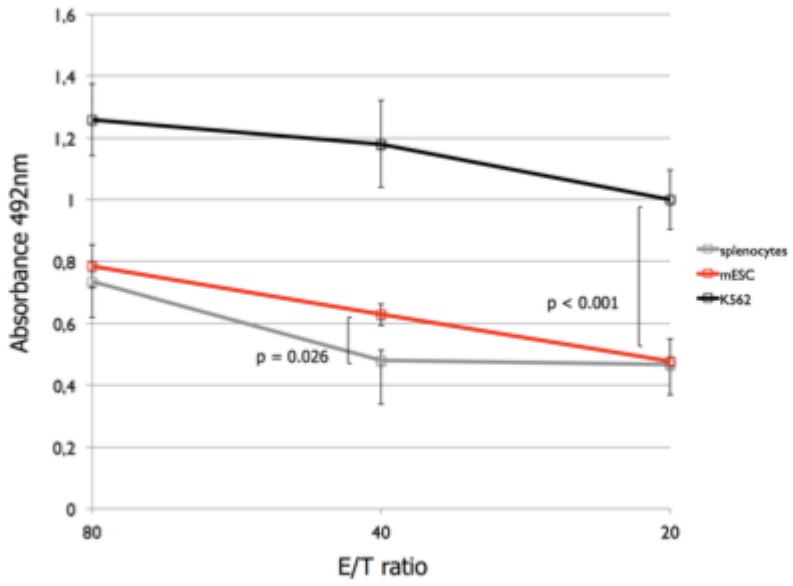

Figure 20. NK cell lysis using LDH. The spleens of Balb/C_nu/nu mice were harvested. NT-ESC (target cells) were plated in V-bottom 96-well plates (Nunc) at 10^4cells/well and mixed with splenocytes (effector cells) at different ratios (1:20,1:40, 1:80). LDH-levels were measured using a TECAN sunrise absorbance reader. Curves of K562 (black), mESC (red) and splenocytes (gray) are shown. Data represent the mean±SD of triplicate samples.

5.6.5 BLI of anti-asialo GM1 treated animals

I.v. injection of anti-asialo GM1 antibody exceeds the complexity of an i.p. injection. Since i.p. treatment also showed a reduction of NKG2D+, CD3- splenocytes by FACS, compared to untreated mice (i.v.: NKG2D positive cells:

untreated: 5.6% of total splenocytes; treated: 1.2% of total splenocytes / i.p: NKG2D positive cells: untreated: 6.89% of total splenocytes; treated: 3.2% of total splenocytes) we stopped i.v. injection of anti-asialo GM1 antibody. [Figure 21].

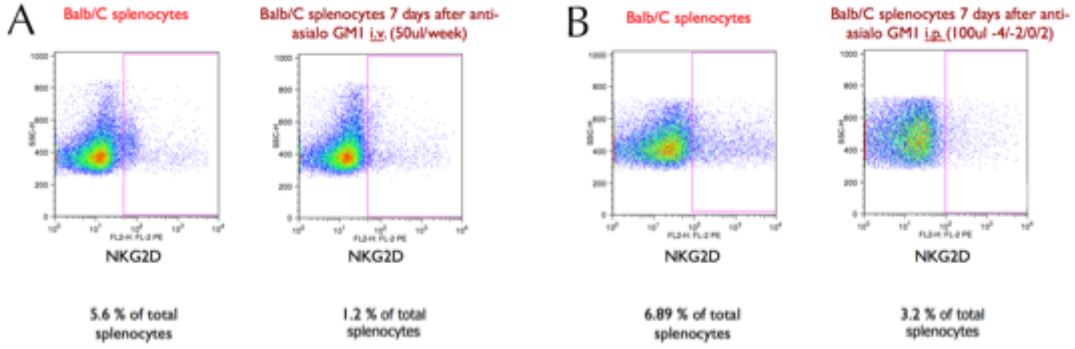

Figure 21. NK-cell reduction after anti-asialo gm1 treatment. Balb/C mice were either i.v. (A) or i.p (B) injected with 100µl of anti-asialo gm1 antibody. After 7d spleens were harvested. Splenocytes were analyzed for NKG2D expression, commonly expressed by NK-cell.

On day 0,1 and continuously every other day BLI-measurements were performed (n=3). One animal in the mitochondria-incompatible group showed a formation of a tumor, which was hard in its consistence, but was later rejected (d47) and 2 animals rejected the cells entirely (d19, d23). Interestingly, none of the nucleus-incompatible animals showed signs of rejection, as 2 animals formed tumors and were euthanized due to tumor volume and one animal showed no signs of rejection or tumor growth, as the signal increased up to day 27 and then remained static up to day 45. At this point in time we stopped further BLI-Measurements. One animal in each group had to be excluded, as it died due to the i.p injection or anesthesia.

5.6.6 BLI of Balb/C_nu/nu mice

Next to anti-asialo gm1-mediated reduction of NK-cells, we used Balb/C_nu/nu mice (n=5) to investigate the role of further subsets of the immune system that do not rely on T-cells. These mice lack a thymus and are therefore unable to produce functional T-cells. They carry the genetic background of Balb/C mice so we used them as a subgroup in the mitochondria-incompatible group. After the injection of 5x10^6 luc+ NT-ESCs, Balb/C_nu/nu mice showed a rapid increase in signal intensity measured by BLI [Figure 22]. Starting on day 11, tumor formation became visible through the thin skin of the animals and was

constantly increasing until measurements were stopped on day 25. At this point in time we could not observe any signs of rejection and the tumor size exceeded justifiable hindering of animal life. These data imply, that in the absence of functional T-cells, the immune system was not capable of rejecting NT-ESCs carrying allogeneic mtDNA. Definite conclusions about the contribution to the rejection process of different subsets in the animal's immune system have to be evaluated critically. Further analyses of cytokine production via ELISPOT would help to gain a deeper insight.

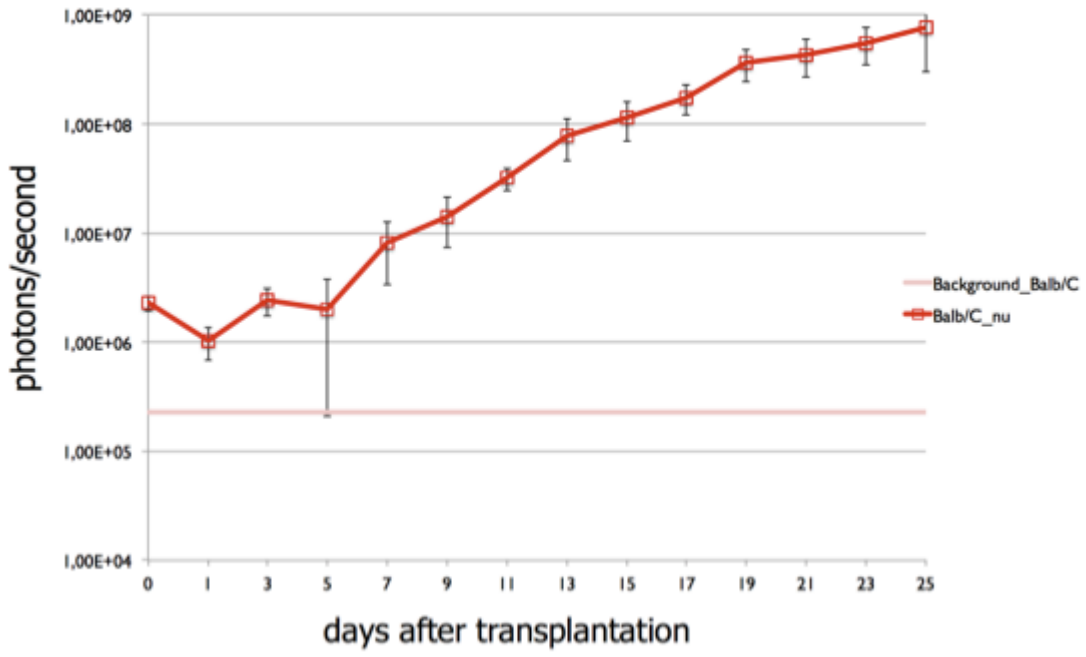

Figure 22. Cell survival in T-cell deficient Balb/C_nu/nu mice. Balb/C_nu/nu mice were injected with $5x10^6$ fLuc+ NT-ESCs (n=5). Graph represents cell survival *in-vivo* by logarithmic photon emission per square centimeter per steradian (*y-axis*) against days of imaging (*x-axis*).

5.7 Immune response towards ESC derived Cardiomyocytes

With differentiation into cardiomyocytes MHCI level expression rose as well as the activity of mitochondria. We assumed that CMs trigger a stronger recognition of the immune system compared with NT-ESCs, as their antigenicity should rise with an increase of mitochondrial activity and MHCI level expression. ELISPOT assay revealed a significant increase of IFN-y, IL-4 and IL-17

secretion in both mitochondria- and nuclei-incompatible animals in comparison to NT-ESC injected animals (mitochondria-incompatible group: IFNγ: 211.1±88.1 p=0.016, IL-4: 217.33±111 p<0.001, IL-17: 19.2±10 p<0.001, n=6; nuclei-incompatible group: IFNγ: 217.15±71.5 p<0.001, IL-4 105.7±59.6 p<0.001, IL-17: 8.67±4.9 p<0.001; n=6) [Figure 23]. Noticeably, mitochondria-incompatible animals displayed a strong IL-4 production against differentiated cardiomyocytes. Additionally, these animals showed increased IgM levels when compared to normal NT-ESC (MFI: 77.87±17.02, p=0.28). Nucleus-incompatible animals did not show elevated IgM levels (MFI: 42.95±6.41, p=ns) [Figure 23]. These data suggest an increase in antigenicity with differentiation into cardiomyocytes.

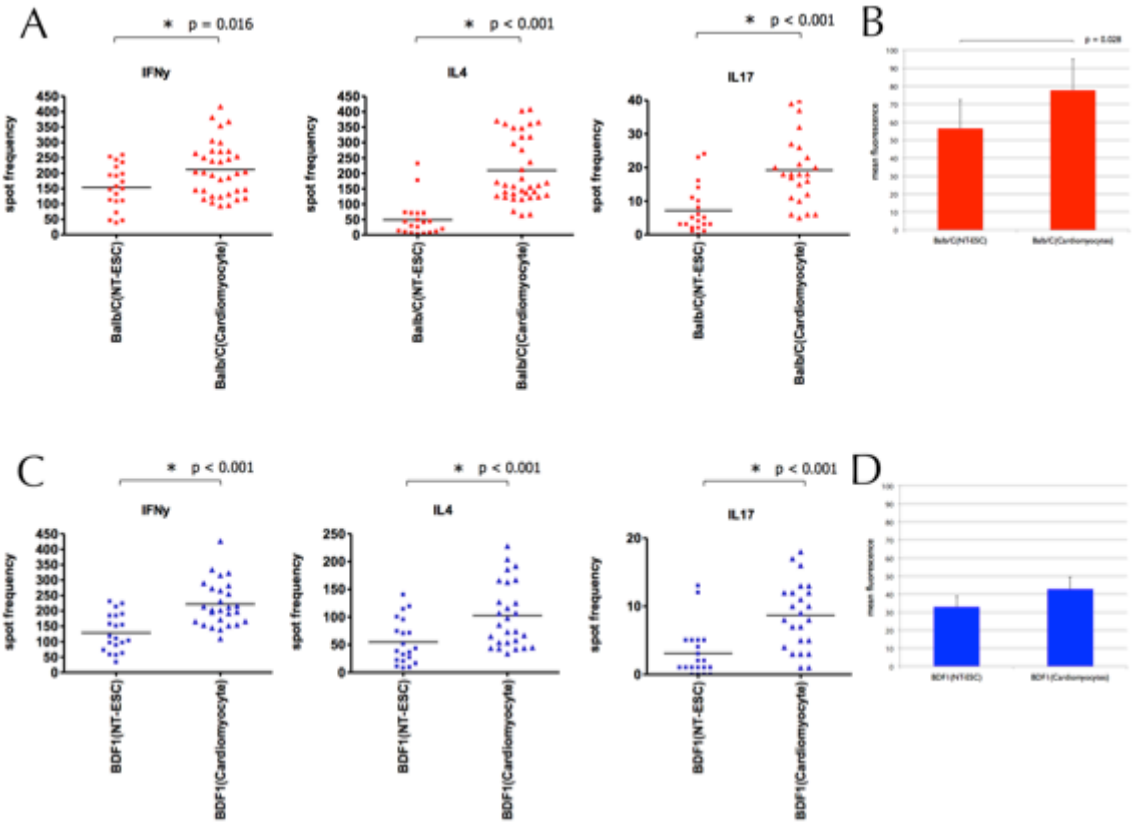

Figure 23. Immune response towards CMs. Mitochondria (A-B) and nucleus-incompatible (C-D) animals were injected with $1x10^6$ CMs. Spleens and sera were harvested after 5d. Cellular (A/C) immune response was investigated via ELISPOT and compared to native NT-ESCs. Donor specific IgM-production was observed in animal sera (B-D) and also compared to native NT-ESCs. Two-way ANOVA *posthoc* Bonferroni was performed for statistical analysis.

5.8 Strain dependent ELISPOT

We performed ELISPOT analyses to investigate the cytokine production of both immune competent mice strains used within this study. This assay aimed to reveal any differences in the strength of the strains' immune systems. Both mice strains received splenocytes of the other strain, which we also used as the stimulator *in vitro* (n=5). To clarify, BDF1 mice were injected with Balb/C splenocytes and vice versa. ELISPOT results showed that BDF1 mice secreted significantly more IFNγ compared to Balb/C mice (p=0.06). IL-4 production did not significantly differ between these strains [Figure 24].

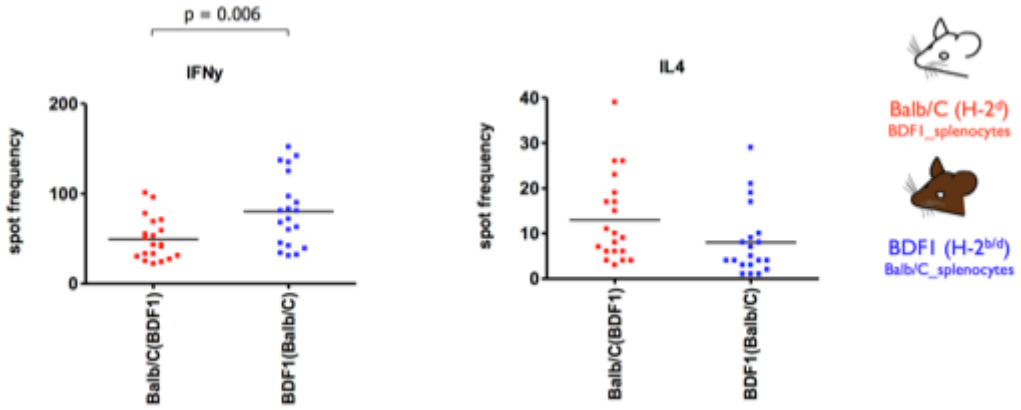

Figure 24. Direct allorecognition ELISPOTS. . 1x10^6 Balb/C-splenocytes were injected in BDF1 mice or 1x10^6 BDF1-splenocytes were injected in Balb/C mice. Systemic immune response was investigated after 5d with unidirectional ELISPOT assays. IFNγ and IL-4 production was observed between Balb/C and BDF1 mice. Spots were automatically enumerated using an ELISPOT plate reader (CTL). Student's t-test was performed for statistical analysis.

6 Discussion

At first glance, ESCs seemed the perfect source for regenerative medicine as they can differentiate into all cell types of the three germ layers and were believed to be immunologically privileged (Tian et al., 1997, Draper et al., 2002, Drukker et al., 2002). However, many groups disproved the last thesis, as ESCs were rejected in allogeneic/xenogeneic settings underlined by a severe infiltration of various cell types of the adaptive and innate immune system. NT-ESCs have been envisioned as an approach to generate patient-matched ESCs. Additionally another hurdle was taken when optimized SCNT approaches allowed derivation of human NT-ESCs (Tachibana et al., 2013). However, uncertainty remains whether foreign mitochondrial do require immunosuppression in clinical settings.

In this study, histocompatibility of SCNT generated mESCs was tested in order to investigate if cloning may be able to play a stronger role as a source for pluripotent stem cells regenerative medicine. We wanted to compare mitochondrial and nuclear antigens on the level of mHCs. Therefore, the nucleus and oocyte for the SCNT were selected to match the study design. A Balb/C nucleus, carrying the MHCI haplotype H-2d, was injected into the oocyte of a BDF1 mouse. Resulting NT-ESCs should thus possess the H-2d haplotype, as MHCI molecules are encoded by the nucleus (Gruen et al., 1996). BDF1 mice show both H-2d and H-2b MHCI haplotypes. Cells that carry the H-2d haplotype are unlikely to be recognized by the immune system of BDF1 mice, because of MHCI molecules. Furthermore, we differentiated NT-ESCs into cardiomyocytes to elucidate changes in antigenicity properties due to the higher maturity of transplanted cells. ELISPOT, DSAs and BLI assays explored involvement of the adaptive immune system in the rejection of NT-ESC with allogeneic mtDNA. To investigate further subsets of the immune system we performed a cytotoxicity assay and BLI measurements of either anti-asialo GM1 treated animals or Balb/C_nu/nu.

6.1 Are ESCs recognized by the recipient immune system?

Data presented in this study suggests that ESCs are recognized by the host immune system regardless of their nuclear and mitochondrial genetic background and other groups underline these findings (Swijnenburg et al., 2008, Ishikawa et al., 2010). Thus, the historic belief in immune-privileged embryonic stem cells (Tian et al., 1997, Draper et al., 2002, Drukker et al., 2002) needs to be questioned.

Immunological assays implied in this study lead to the assumption that allogeneic mHCs are equally or even more potent if encoded by the mitochondrial and not the nuclear genome. BLI-Measurements showed complete rejection in mitochondrial-incompatible animals, whereas within the nucleus-incompatible group more than half of the animals tested could not control the tumor growth. Tumor formation is used to determine if ESCs are rejected or tolerated by the host's immune system (Ishikawa et al., 2010; Swijnenburg et al., 2008). Clearly, as animals were euthanized for ethical reasons, spotting a possible immune response that might have eventually controlled the tumor growth was not possible. This impediment has already been addressed by other groups (Koch et al., 2008, Utermöhlen et al., 2009).

6.2 Can allogeneic mitochondria act as mHCs in ESCs?

The existing literature cannot give definite predictions as to whether rejection or tolerance is to be expected. As mtDNA only carry a few alleles, it has been proposed that the risk of an immune response to allogeneic mitochondrial antigens might be low (Drukker, 2008). In cows, nuclear-transfer-generated cells and tissues could be transplanted without rejection and remained viable in the nuclear donor animal, even though their mtDNA haplotype differed (Lanza et al., 2002). Recently however, another group was able to show that cells carrying foreign mtDNA generated via fusion were detected and rejected by the innate immune system of nuclear donor hosts (Ishikawa et al., 2010). However, as different assay conditions, animal models and methods in ESC generation were used in these two studies, precise conclusions cannot be drawn.

In this study NT-ESCs were detected and rejected by the immune system of the nuclear donor. ELISPOT, DSAs and BLI-measurements show immunological reactions towards foreign mtDNA, as to be cytokine-secretion, antibody production or destruction of living cells. As all polymorphic proteins should be considered “immunogenic”, it is generally assumed that a stronger immune response occurs against allogeneic nuclear rather than mitochondrial proteins because nuclear encoded proteins outnumber those of the mitochondrion. However, the presented data shows a rather different conclusion.

In ELISPOT no differences in cytokine-production (IFNγ, IL4, IL17) were observable between mitochondrial- and nuclear-incompatible animals for direct and indirect allorecognition. However, in indirect allorecognition mitochondrial incompatible mice secreted significantly more IFNγ than the SCID beige mice, whereas the nuclear-incompatible group was insignificant. This could indicate a difference in antigen presentation of APCs between mitochondrial and nuclear antigens, either in the velocity and/or the antigen’s strength.

To activate the humoral immune system, one of the major pathways is via IL-4 secretion of Th-2 cells. IL-4 stimulates B-cells proliferation as well as B-cell antibody class switching (Murphy et al., 2008). For donor specific antibodies, insignificant differences of circulating IgM-antibodies were observed when mitochondrial and nuclear-incompatible groups were compared. Both groups had an activation of cells contributing to the humoral immune pathway, even though this was not significantly visible in their IL-4 secretion. However, this might be explained as the negative control showed a relatively high IL-4 cytokine production, which we used as the reference. A spontaneous production of both T-cells and immunoglobulin in immunodeficient SCID beige, known as “immune leakiness”, has been addresses by other groups (Mosier et al., 1993, Green et al., 1999) and possibly explains the observed IL-4 levels in SCID beige mice. Additionally the Balb/C background is known as a prototypical Th2 strain (Mills et al., 2000), which might contribute to the measured IL-4 production in our SCID beige mice as they hold the Balb/C background. Detectable IgG antibodies at day 15 strengthened the conclusion that activated B-cells are present and stimulated over a certain period, given that Immunoglobulin class switching from IgM to IgG does need time and particularly

stimulation via IL-4 and IFN-y (Murphy et al., 2008). Yet, the contribution of allospecific antibodies to the whole rejection process remains uncertain. Both nucleus-incompatible and mitochondrial-incompatible animals showed a high presence of donor specific IgG-antibodies. However, just the mitochondrial-incompatible animals were able to fully control and reject transplanted NT-ESCs in BLI measurements.

ELISPOT and DSA analysis only demonstrate a “snapshot” rather than a longitudinal assessment of transplanted cell survival over time (van der Bogt et al., 2006). Due to these limitations, conflicting results may be reached for signs of rejection. To avoid these problems NT-ESCs were stably transduced with a gene construct encoding for fLuc under the control of a CMV promoter. BLI-measurements of recipient animals allow comprehensive none invasive *in vivo* cell tracking. Instead of just observing tumor size development (Ishikawa et al., 2010), BLI also detects small numbers of cells, thus giving a better indication of cell proliferation. This method is also completely objective. All mice injected with cells carrying allogeneic mitochondrial mHCs, eventually rejected transplanted cells. In contrast, only 3 out of 7 animals that received cells with nuclear encoded mHCs rejected transplants, the rest formed a tumor. These findings tend to give mHCs, which stem from mtDNA, a stronger antigenicity than those from nuclear DNA. Similar results were observed by a group in Japan, however they looked at cells derived via fusion (Ishikawa et al., 2010).

Because of increasing evidence of differences in immune responses between strains (Playfair, 1968, Sellers et al., 2012), both inbred stains, Balb/C and BDF1, were studied. BDF1 mice showed a higher IFNγ secretion compared to Balb/C mice, whereas IL-4 production did not differ significantly. Balb/C mice are known to have a propensity towards the Th2 pathway, whereas mice coming from the C57Bl/6 are more Th1 prone (Müller et al., 1993, Hsieh et al., 1995). Thus, tumor formation in BDF1 mice (C57Bl/6xDBA/2) seemed unlikely because of a weaker immune system compared to Balb/C mice. Differences in time needed to reject NT-ESC can be explained, since ESCs can differentiate into all cell types which themselves carry diverse immunological properties, which in turn can result in either a faster, stronger, slower or weaker immune rejection (Slukvin et al., 2006, Drukker, 2008)

In conclusion, mitochondrial encoded mHCs are possible immunogenic antigens. They lead to an activation of the cellular and humoral immune system resulting in active rejection of transplanted cells. Data collected in this study indicates at least a similar immunogenicity of mitochondrial and nuclear mHCs.

6.3 What happens to antigenicity if ESCs are differentiated?

Classic MHCI-molecules are present on almost all nucleated cells within the mammalian body. After cellular and extra-cellular proteins are processed they are presented via MHCI-molecules to circulation T-cells. One of the fundamental mechanisms to distinguish between self and foreign is based on the interaction between the T-cell receptor and the MHC class I molecule. A mismatch of the MHCI-molecule leads to the rejection of the transplants, since the MHCI-molecule itself is highly immunogenic. When cells differentiate, immunological surface markers are up regulated. Hence a higher immunogenicity of ESC derivatives is plausible (Drukker et al., 2002, Drukker, 2008).

MHCI expression of NT-ESCs did increase with differentiation. While CMs showed a 30-fold of MHCI, IFNγ-stimulated NT-ESCs showed only a 7-fold increase. In contrast, MHC class II molecules (MHCII-molecules) are constitutively expressed by antigen presenting cells (B-cells, dendritic cells and macrophages). NT-ESC did not show any levels of MHCII-molecules present on the cell surface, which is plausible, as they are not believed to act as antigen presenting cells. Furthermore, MHCII induction with IFNγ has not been seen (Drukker et al., 2002). Only with differentiation into hematopoietic and DC lineage an increase of these molecules was observed (Slukvin et al., 2006, Senju et al., 2007). Though it seemed unlikely to observe an increase of MHCII levels in our setting. To fully validate that MHCII-molecules are absent on NT-ESCs and differentiated cardiomyocytes, MHCII expression should be measured under the presence of IFNγ in further studies.

For this study, a further postulation was helpful as differentiation also contributes to a higher density of mitochondria per cell (Mummery, 2010, St.

John et al., 2010). Mitochondria amounts widely change between different mature cell types as well as during embryogenesis (St. John et al., 2010). The density of mitochondria per cell often indicates the cell's requirement for ATP production (Moyes et al., 1998). ESCs show small amounts of mtDNA copy numbers, approximately 30-45 copies/cell (Facucho-Oliveira et al., 2007) as they mainly rely on an anaerobic metabolism (Cho et al., 2006, Chung et al., 2007). Although, glycolytic energy production seems to sufficiently meet the demand of undifferentiated ESCs (Cho et al., 2006, Chung et al., 2007), successful differentiation requires an activation of the mitochondrial aerobic metabolism (St. John et al., 2005, Chung et al., 2007). Accordingly, a differentiation of immature mitochondria of undifferentiated ESCs into a more mature and active mitochondrial network has been postulated in fully differentiated cardiomyocytes and neurons. They expressed high levels of nuclear- and mitochondrial-encoded electronic transport chain subunits, increased their ATP production via OXPHOS (Chung et al., 2007) and showed an enriched mtDNA content per cell (Filser et al., 1997, Miller et al., 2003, Mummery, 2010). In this study TMRM was used to visualize mitochondrial membrane potential ($\Delta\psi$) as its signal represents only the probe accumulated in mitochondria (Floryk and Houstek, 1999) and has been correlated with mitochondrial activity (Baracca et al., 2003). Our TMRM measurements of NT-ESC, teratoma cells and cardiomyocytes showed an increase in $\Delta\psi$ with differentiation. Greater mitochondrial activity has additionally been linked to an enriched mitochondria amount per cell (Filser et al., 1997, Miller et al., 2003, Mummery, 2010). We then suggested an increased mitochondria amount with cardiomyocytes differentiation in our study, since $\Delta\psi$ rose. Accordingly, it has been shown that out of all of the differentiated cell types an organism carries, cardiomyocytes have the highest amount of mtDNA copy number per cell (Mummery, 2010).

We hypothesized that a higher presence of MHCI molecules and an increase in the activity of mitochondria render cells more susceptible towards lymphocytes. The next step was therefore to examine whether immune cells recognize CMs more strongly compared to their pluripotent partners.

ELISPOT results revealed a stronger release of all cytokines (IFNγ, IL4 and IL17) in CMs compared to undifferentiated NT-ESCs in all groups. The extensive elevation of IL-4 production in mitochondrial-incompatible animals was unique and correlated with a higher presence of IgM antibodies in animal sera. Although nuclear-incompatible mice also showed a significant increase in IL-4 production, levels of IgM antibodies did not increase. This can be explained since the Balb/C mouse favor Th2 cytokine production with low IFNγ and high IL-4 levels in comparison to other members of its specie (Mills et al., 2000). A rather different conclusion could be that foreign mitochondria mainly trigger the humoral pathway. This could be true, because of the increased mitochondrial activity and the coherent postulated gain in mtDNA copies, whereas the mononuclearity remains. To verify this assumption, comprehensive ratios of mitochondrial and nuclear encoded proteins inside both cell types should be analyzed in further studies.

In summary, differentiation increases the immunogenicity of ESCs. Higher responses are visible in the cellular and humoral immune system. It is arguable that mitochondrial antigens produce a stronger response than polymorphic nuclear encoded proteins. However, the underlining mechanisms have to be analyzed in future studies.

6.4 Which cells seem to play a role in the rejection process?

Data collected in this study, revealed an immune recognition of transplanted NT-ESCs by Th1-, Th2-cells due to allogeneic mitochondria and nuclei. Re-injecting animals with NT-ESCs resulted in a stronger cytokine-release in ELISPOT for IFNγ, IL4 and IL-17, highlighting a T-cell dependent immune response. Never seen foreign molecules are mainly detected by the adaptive immune system and are recognized better when seen for the second time (Murphy et al., 2008), which leads to the assumption that foreign mitochondria were detected by the adaptive immune system.

The innate immune system is mainly focused on antigens present throughout evolution. Ishikawa et al. postulated that cells with different mitochondrial and

nuclear backgrounds are lysed and rejected by the innate immune system. NK-cells were deemed responsible for mediating this process (Ishikawa et al., 2010). So we wanted to augment our study and investigate T-cell independent parts of the immune system.

Balb/C_nu/nu mice lack a thymus and a therefore unable to produce functional T-cells. This property makes them very useful when studying differences in T-cell dependent and independent parts of the immune system (Sundstrom and Cherniak, 1993). Their immunological capabilities are unlikely to involve conventional mature T lymphocytes (Kiessling et al., 1975). Cytotoxic assays revealed a rather low antigenicity of NT-ESCs with foreign mitochondria to Balb/C_nu/nu splenocytes. In our assay we used K562 cells. They lack MHCI-molecules, rendering them optimal targets for NK-cell lyses as the MHC complex is required for the inhibition of NK cell activity (Lozzio and Lozzio, 1979). Though it is questionable whether human K562 cells are an accurate target for murine NK cells and are able to act as the best positive control in this assy. While the signaling pathways regulating B and T lymphocytes are well conserved between humans and mice, those for NK cell activation might differ between these two species (Colucci et al., 2002). However, Colucci et al. also reviewed that the divergence could be deceptive. In accordance, CD94/NKG2 expressed by human and mice (Di Santo, 2006), has been shown to bind to the murine non-classical MHCI molecule Qa-1b, which shares several features with HLA-E (Salcedo et al., 1998). However, Balb/C_nu/nu splenocytes seemed capable to lyse human K562 cells, as we saw increased LDH levels for splenocytes with K562 cells in comparison with only K562 cells or splenocytes.

Balb/C_nu/nu mice were unable to reject NT-ESCs in BLI measurements and all animals showed tumor growth. Furthermore, BLI-measurements of animals with reduced NK-cells due to anti-asialo gm1 treatment did not show remarkable differences to animals with an intact immune system. However, the treatment with anti-asialo gm1 antibodies was recently identified to suppress basophils, which raised concerns about possible contribution of further immune cells, rather than or in addition to NK cells, to some observations in anti-asialo gm1 treated mice (Nishikado et al., 2011).

The data retrieved by Ishikawa et al. competes against data collected in this study. Differing methods and cell types used might explain some of these differences. Ishikawa et al. measured rejection or tolerance towards transplanted cells mainly by tumor growth, a method that is arguably subjective. Data collected from flow cytometry analysis of infiltrating cells derived at d12 revealed CD3+ positive cells, suggesting the adaptive immune system was activated. However this was not further discussed in the paper.

Despite some debatable assay conditions, data collected in this study led to the assumption that T-cell independent parts of the immune system are relatively unimportant for rejection processes of ESCs carrying allogeneic mitochondria. Instead the adaptive immune system seems capable of remembering foreign mitochondria.

6.5 Regardless of their genetic background, are ESCs immunogenic?

Although much of the current literature is focused on a direct immune rejection due to allogenicity recent papers have demonstrated the importance of expressed pluripotency markers in mediating graft rejection. As mentioned before Sox2, Oct4, Nanog, SSEA3, SSEA4, Klf4, TRA-1-81 and TRA-1-60 are highly expressed in undifferentiated pluripotent cells and play a crucial role in mediating the pluripotent state these cells. Under normal circumstances autoreactive T cells that can develop through high recombination within the V(D)J region of the T-cell receptor gen are suppressed and forced towards apoptosis through thymus-mediated negative selection (Nitta et al., 2008). The fetal thymus becomes capable of negative T-cell selection by approximately 7 weeks of gestation (Res and Spits, 1999) four weeks after the last pluripotent cells have differentiated. Therefore it is unclear whether pluripotency markers were ever presented to T-cells and had the possibility to prime them. Pluripotency antigens include glycans, proteins and other unique cell surface molecules, which are part of the core transcriptional network of hESCs. It is important to realize that possible T reaction against pluripotency markers would

dramatically decrease the potential of regenerative medicine based on pluripotent cells, because it would also exist in an isogenic setting. Recent publications have highlighted immunologic responses triggered by embryonic antigens, which were re-expressed in a variety of oncofetal tumor lines (Siegel et al., 2003, Li et al., 2009, Dhodapkar et al., 2010). Dhodapkar et al. were able to detect OCT4-specific T cells in T cells isolated from over 80% healthy donors. These cells could be expanded in culture using OCT-4 derived peptides-loaded dendritic cells. In conclusion, these data demonstrated a lack of immune tolerance towards the pluripotent markers. Yet, it is unlikely that ESCs, once differentiated, retain embryonic antigens. Hence, a clinical translation will be undisturbed.

Despite all efforts in cloning and ESC transplantation, different strategies to overcome immunological barriers for clinical translations are also currently in the spotlight of modern research facilities. These are classical immune suppression, induction of pluripotency via viral transduction and generation of low-immunogenic stem cells. One ethical aspect of the last two strategies is very appealing in that no further embryos need to be destructed in order to gather ESCs. Yet, none of these strategies enables a conversion of ESCs into clinical settings, as tumor formation and sufficient differentiation have to be mastered first.

7 Synopsis

Somatic cell nucleus transfer (SCNT) was used for embryonic stem cell (ESC) extraction. The nucleus of a somatic cell of the Balb/C strain was transferred to the cytoplasm of an enucleated egg of the BDF1 strain. Resulting ESCs inherited a Balb/C nucleus and BDF1 mitochondria. Therefore they were either incompatible due to their nucleus or mitochondria when transplanted into the Balb/C or BDF1 strain. Immunodeficient SCID beige mice were used as negative controls, whereas BDF1 mice received Balb/C-ESCs for the positive control.

Interestingly, in ELISPOT assays for Th1, Th2, and Th17-cells, foreign mitochondria caused a similar immune response compared to ESCs with foreign nuclear DNA, both being significantly higher than in SCID beige ($p<0.001$). Similar results were observed for donor specific antibodies, highlighting the humoral response**.** Re-injection of ESCs resulted in an adaptive immune response, highlighting the role of T-cell mediated rejection. Potent antigenicity of mtDNA was also observed in longitudinal *in-vivo* cell survival BLI measurements. Antigenicity of ESCs increased with their differentiation into SCNT-ESC-derived cardiomyocytes (CMs), correlating with increased MHCI-expression and greater mitochondrial activity. Mitochondrial activity was visualized using the TMRM assay.

In conclusion, even though foreign mtDNA codes only 13 proteins, its transplantation is sufficient to trigger an adaptive immune response and the production of donor specific antibodies. With differentiation into CMs, mitochondrial activity and MHCI expression increased per cell, which lead to a higher immune response. Therefore, contrary to former beliefs SCNT-derivatives carrying foreign mtDNA are not capable of circumventing the host's immune system as they are recognized and rejected after transplantation, resulting in the need for immunosuppression for potential clinical applications.

8 Literature

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9 Appendix

9.1 Abbreviations

$\Delta\psi$	mitochondrial membrane potential
AEC	Avidin-Enzym-Conjugate
APCs	antigen presenting cells
ASCs	adult stem cells
Balb/C-ESCs	Balb/C embryonic stem cells
BLI	Bioluminescence Imaging
C.T.L	Cellular Technology Limited
CMs	NT-ESC derived cardiomyocytes
COI	cytochrom c oxidase subunit 1
cTL	cytotoxic T-Lymphocytes
D-Loop	displacement loop region
DCs	dendritic cells
DMSO	Dimethylsulfoxid
DSAs	donor specific antibodies
EB	embyroid body
ELISPOT	Enzym-linked immunosorbent spot
ESCs	embryonic stem cells
FACS	Fluorescence-activated Cell Sorting
FCS	fetal calf serum
H-Y antigens	Y-chromosome genes
H.E.	Hematoxylin-Eosin
hESCs	human embryonic stem cells
HLA	human leukocyte antigen
HRP	horseradish peroxidase
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
ICM	inner cell mass

IFNγ	Interferon-γ
IL	Interleukin
IVC	individual ventilated cages
LIF	leukemia inhibitory factor
MEF	murine embryonic fibroblast
mESCs	murine embryonic stem cells
MHC	major histocompatibility complex
mHC	minor histocompatibility complex
mtDNA	mitochondrial deoxyribonucleic acid
mtSSB	mitochondrial single-stranded DNA-binding protein
NAO	nonyl acridine orange
ND1	NADH dehydrogenase subunit 1
NK cells	natural killer cells
NT-ESCs	SCNT derived embryonic stem cells
OXPHOS	oxidative phosphorylation complexes
PAS	periodic acid schiff
PFA	Paraformaldehyde
POLG	Polymerase γ
ROI	region of interest
RT	room temperature
SCID	severe combined immunodeficient
SCNT	somatic cell nucleus transfer
SD	standard deviation
SSEA	stage-specific embronic antigen
TCR	T-cell receptor
TFAM	mitochondrial transcription factor F
Th-cells	T-helper cells
TMRM	tetramethylrhodamine methyl ester perchlorate
umif	UKE Microscopic Imaging Facility
UV	Ultraviolet

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PUBLICATIONS

BOOKCHAPTER

„Immunogenicity of Stem Cells"
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ABSTRACTS

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„Immunobiology of SCNT Derived Embryonic stem cells: Pivotal Role of mtDNA in Rejection of Transplanted Stem Cells"
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- Albert Einstein

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11 Affidavit

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Unterschrift: .....