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# Potential of Heterotopic Cardiac Transplantation in Mice as a Model for Elucidating Mechanisms of Graft Rejection

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## 1. Introduction

Cardiac transplantation displays a well established therapeutic procedure for different end-stage heart diseases. Effective immunosuppressive drugs, progress in operative techniques, modern perioperative intensive care, and application of increasingly potent antibiotics in case of postoperative infections led to an improvement in short term outcome of organ transplantation. Nevertheless, achievements in long term transplant results are rare due to rejection of allografts. Chronic graft rejection is the major cause of late transplant failure.

According to present knowledge, T cells, infiltrating monocytes and macrophages, and NK cells, respectively, are involved in acute and chronic rejection. Numerous clinical trials and investigations in animal or cell culture models point out, that cell adhesion molecules, cytokines, and chemokines play a decisive role in the acute and chronic rejection of solid organ grafts.

Heterotopic cardiac transplantation in mice is considered to be the best model to study immunological mechanisms of transplant rejection. This technique allows the analysis of rejection processes in different mouse strains with defined genetic defects. Thus, distinct immunological receptors and ligands can be scrutinized for their impact on physiological and pathophysiological mechanisms of acute and chronic graft rejection. Results achieved from this model could be transferred to human beings in the majority of cases. As such, the model of heterotopic cardiac transplantation in mice has the potential to discover new therapeutic strategies which can be transferred to the clinic.

The aim of this chapter is to present our comprehensive microsurgical expertise on this model to other research groups in the field of cardiac transplantation. It delineates the practicable microsurgical model of heterotopic cardiac transplantation in mice as performed in our centre, based on the initially presented technique nearly four decades ago. Furthermore, the necessity of this *in vivo* model for a detailed understanding of the underlying mechanisms of transplant rejection is going to be discussed.

## 2. History and clinical-experimental development of transplantation

Basic requirement for transplantation of vascularized organs was the development of surgical vascular anastomosis techniques, crucially brought forward by Alexis Carrel in Lyon, France at the beginning of the last century [Carrel & Guthrie, 1905]. He successfully performed numerous heart transplantations in dogs and received the Nobel Prize for his research in 1912. In the year 1954 Joseph Murray (Boston, USA) performed the first successful solid organ transplantation in human beings in terms of the first kidney transplantation between monozygotic twins [Merrill et al., 1956]. In 1963 both Starzl (Denver, USA) and Hardy (Mississippi, USA) performed the first liver transplantation and lung transplantation, respectively. The first pancreas transplantation was realized by Richard Lillehei (Minnesota, USA) in 1966 and finally Christian Barnard (Cape Town, South Africa) performed the first human heart transplantation in 1967.

The knowledge about transplant immunology is derived from basic experimental findings during transplantation in animal models. Cellular principles of organ rejection were conclusively described by the English biologist Peter Medawar, who received the Nobel Prize for the “discovery of immunological tolerance” in 1960 [Billingham et al., 1951]. In 1953, histocompatibility antigen was reported for the first time on the surface of leucocytes. Jean Dausset therewith established the basis for histological typing between donor and recipient which is nowadays routinely performed within the pre-operative screening previous to each transplantation, the so-called HLA matching (testing of congruousness of those genes that are responsible for transplant rejection). Nevertheless, T cell mediated immune defence against the graft is not covered in these investigations though T cells take up a key function in transplant rejection [Sayegh & Carpenter, 2004]. This T cell reactivity could be accurately examined in our transplant centre in living donor kidney transplantation between monozygotic twins and in consequence immunosuppression was first reduced and then completely withdrawn [Hüser et al., 2009]. To date, medication free development of tolerance or at least reduction of required immunosuppressive drugs remains to be the unchanged goal for increased postoperative transplant survival.

Of course, transplantation of organs between genetically identical individuals continues to be exceptional as transplantation is usually performed in an allogeneic context which implicates the risk of an acute rejection episode. Technical advance and improvements in medicamentous therapy by implementation of potent immunosuppressants lead to an overall one-year graft survival of more than 80% [Christie et al., 2010]. On the other hand, no relevant enhancements in long-term outcome of grafts could be determined. Chronic transplant rejection is hereby the main reason for late graft failure. Besides non-immunological donor- and recipient-dependent factors, mainly immunological reactions represent an important role for graft survival. Heterotopic heart transplantation in mice provides an important and valid model for analysis of immunological events during acute and chronic rejection mechanisms.

## 3. Necessity of an *in vivo* model for investigation of immunological events mediating organ rejection

The reaction against an allograft is composed of a complex cascade of immunological processes and some parts of them can be analyzed *in vitro*. However, detailed investigation

of rejection mechanisms of vascularized grafts includes afferent and efferent steps like e.g. sensitisation of the recipient, antigen processing in lymphoid organs, differentiation and proliferation of immunocompetent cells of the recipient that detect the graft to be “extraneous” and finally direct their movement into the graft. These processes all culminate in graft failure, but they are not reproducible *in vitro*, because conclusion on the exact genesis and therefore identification of the causality of specific interactions is not possible. Experimental *in vivo* investigations are especially necessary to display the dynamic of these processes. The animal model is therefore an ideal compromise between clinical reality and experimental reproducibility. The study of genetically modified rodents has become commonplace within immunological research. Sophisticated advances in gene-altered models make it possible to appoint the function of a certain interaction of specific gene products in the context of graft rejection. Within these gene replacement (knock-in) or loss of function mutation (knock-out), mice have been generally accepted for both technical and nontechnical reasons. An observed phenotype can provide clues to the mechanisms of transplant immunology. Moreover, the use of inducible transgenic systems enables to control the location and time of transgene expression in certain tissues and avoids lethal deletion in knockout mice or compensation by various gene products. For evaluation of immunological and especially transplant immunological questions, results derived from the mouse model could directly be transferred to human beings for the most part, albeit the observations must be interpreted carefully due to some differences in both the innate and adaptive arm of the immune system [Mestas & Hughes, 2004]. A large number of important gene products that play an important role in this context were first defined in the mouse. Nearly all interaction molecules like T cell-receptors, cytokine-receptors, accessory molecules, and cell-activation-markers are characterized and a large number of genetically well-characterized transgenic and knock-out strains of the molecules are available. So far, use of homologous recombination to modify genes in embryonic stem cells was only feasible in mice because of the absenteeism of germline-competent embryonic stem cell lines in other species. Therefore, the murine model is more useful for the investigations of transplant rejection, although it requires a higher level of microsurgical skill than the technique in rats. It is only recently that Tong et al. have demonstrated stem cell based gene targeting technology in the rat [Tong et al., 2010]. Therefore the rat model might provide an adequate, powerful tool for the advancement of our understanding in transplant immunology in the future. The most frequently applied transplant model is still the heterotopic mouse heart transplantation. This procedure was described for the first time by Corry in 1973 [Corry et al., 1973] and the method was comparable to the cardiac transplantation in the rat as performed by Abbott et al. in 1964 [Abbott et al., 1964] and Ono et al. in 1969 [Ono & Lindsey, 1969].

A technique utilizing the transplantation of a non vascularized heart was established by Fulmer and coworkers, where neonatal murine cardiac tissue was placed subcutaneously into the pinna of the recipients ear [Fulmer et al., 1963]. Using this technique certain aspects of acute rejection have been studied. Nevertheless, the factors that lead to cardiac allograft vasculopathy all interact within the transplanted vessels at the blood / endothelial interface, making a vascularized cardiac transplant model imperative for the studies of chronic graft rejection [Hasagewa et al., 2007]. Other research groups refer to a simplified and technically easier model of vascularized transplantation in which the graft is anastomosed to the cervical vessels [Chen, 1991; Tomita et al., 1997]. However, according to Doenst et al., first, the positioning of the transplanted heart in an infrarenal position is self-guided and less

likely to allow torsion, and second, the carotid artery is smaller in diameter than the ascending aorta of the donor, what makes the aorto-aortic anastomosis easier to perform [Doenst et al., 2001]. Finally the decision of which operative procedure is performed depends on the personal preference.

#### 4. Anatomical specialities of the heterotopic heart transplantation model

The harvested donor heart is transplanted heterotopically by performing vascular anastomoses of the aorta and the pulmonary artery to the large infrarenal vessels of the recipient [Corry et al., 1973]. Therefore, the recipient mouse is not dependent on the functioning of the graft as its own heart remains untouched and the mouse undergoes rejection of the graft without impairment of physical well-being. A difference of this method compared to orthotopic heart transplantation is related to the technique of vessel anastomoses. The ascending aorta (AscA) of the donor heart is anastomosed end-to-side to the abdominal aorta (AbdA) and the pulmonary artery (PA) is anastomosed end-to-side to the recipient's inferior vena cava (IVC). This leads to a retrograde blood flow from the abdominal aorta via the ascending aorta directly into the coronary arteries whilst bypassing the left ventricle (LV) and left atrium (LA). After a while a thrombus accrues in the left ventricle. Hence, the oxygen supply of the heart muscle is hereby ensured by the capillary bed. Coming from the coronary arteries, the blood flow then passes the coronary veins and next converges in the coronary sinus (CS) and the right atrium (RA). From the right atrium the blood courses to the right ventricle (RV) and using the stump of the pulmonary trunk it finally reaches the recipients IVC (see *fig. 1*). The presented model is a so-called „non working heart model“, because the graft does neither maintain physiological cardiac output nor pump against physiological pressure.

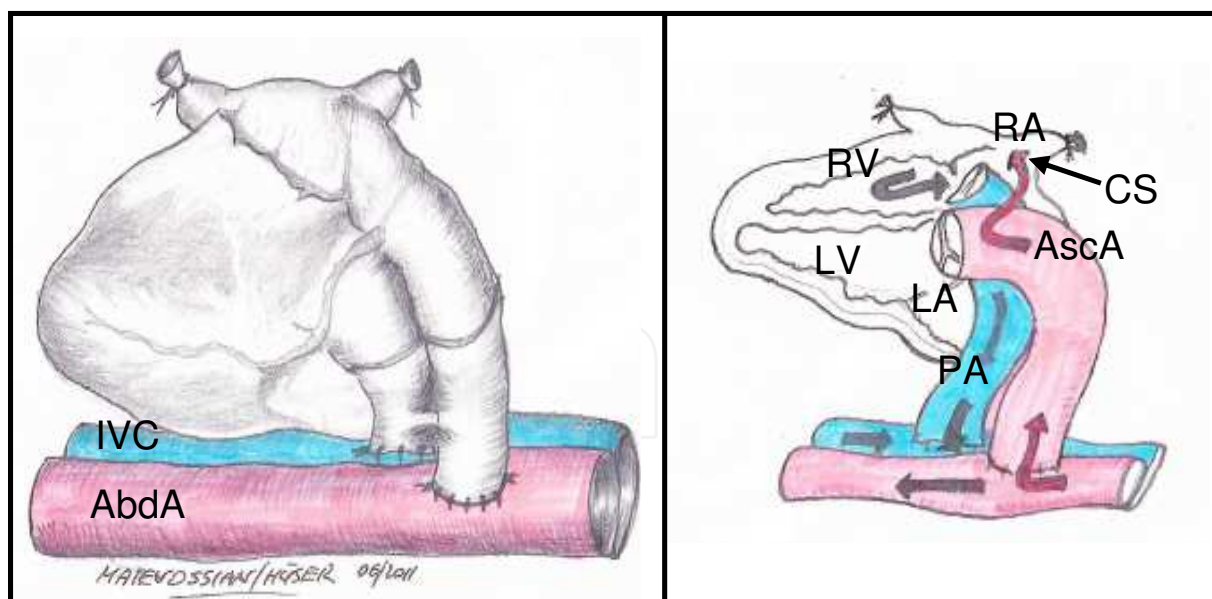


Fig. 1. Illustration of blood flow in heterotopically transplanted heart cardiac grafts

#### 5. Options of rejection diagnostics after heterotopic heart transplantation

A relevant advantage of this operative method is the efficient rejection diagnostic of the transplanted heart. An algorithm on investigative technique could be developed to



guarantee both the most comfortable examination for the transplanted mouse and the complete and comprehensive acquisition of acute and chronic rejection.

Finger palpation of the transplanted heart is a sensitive method to appraise time-dependent course of rejection. Concerning the contraction power and the induration of the graft, respectively, different stages can be graduated [Schmid et al., 1994]. In case of applying too high pressure, the graft cannot fill up and fully pulsate, and contractility is underestimated [Martins, 2008]. In the hand of an experienced diagnostician, slightest differences, especially the rapidly decreasing contraction power during acute rejection can be measured. In diagnostics of chronic transplant failure, evaluation occasionally might be difficult due to successive impairment of cardiac pump functioning, as heart pulsation is directly related to the amount of intact myocard.

The validity of supplementary analysis to get the exact rejection time point remains controversial. Performance of electrocardiogram (ECG) requires exact subcutaneous placement of needle electrodes [Superina et al., 1986], validity is heavily dependent on the organ's position and movement [Mottram et al., 1988], and therefore the utility of this method is limited. In acute rejection, frequency shows rapid decrease in combination with various ECG alterations [Babuty et al., 1996]. In literature, magnetic resonance imaging is described as an additional tool in diagnostics for assessment of transplanted hearts. Nevertheless, this expensive procedure should be reserved for specific settings only. Moreover, in perfusion studies of solid grafts, results revealed to be extremely dependent on length and depth of narcosis [Wu et al., 2004]. Recent investigations describe high-frequency ultrasound biomicroscopy modality besides conventional echocardiography, a new non-invasive imaging method for diagnostic of acute rejection [Bishya, 2011]. Various papers report on a decrease of end-diastolic diameter and an increase of left ventricular posterior wall thickness, respectively, to be parameters of acute rejection. However, changes in left ventricular posterior wall thickness, seem to be increasingly difficult to measure after the fifth post-operative day, which points to the limitation of the use of echocardiogram in diagnosing acute allograft rejection [Scherrer-Crosbie et al., 2002]. In principle, every final rejection, either acute or chronic, has to be confirmed by diagnostic laparotomy to avoid misjudging passive movement of the graft by transmission of the aortic pulsation as graft beating.

## **6. The method of heterotopic heart transplantation in mice**

The model of heterotopic heart transplantation in mice has been varied manifoldly since its first description by Robert Corry and Paul S. Russell in 1973 [Mao et al., 2009; Wang et al., 2005; Hasegawa et al., 2007]. The following chapter describes the transplantation procedure and incorporates our experience using an operating microscope (OPMI-6, Carl Zeiss, Jena, Germany) and a magnification between 4x-20x objective. Operation time is approximately 45 minutes and peri-operative mortality in the hands of an experienced micro-surgeon is less than 5%.

### **6.1 Anaesthesia and analgesia**

All operative procedures in animals are performed using Isoflurane narcosis (Forene with 1-Chloro-2,2,2-trifluoroethyl-difluoromethylether). Both donor and recipient mouse can be operated under pain-free, unconscious, and relaxed conditions. Isoflurane narcosis brings

along the crucial advantage of easy handling and having almost no influence on the animal's blood pressure in contrast to other narcotics such as e.g. Ketanest. Furthermore, Isoflurane narcosis is the least hepatotoxic narcotic drug. Basal anaesthesia is performed with 5% Isoflurane and for maintenance approximately 2% Isoflurane are necessary. Endotracheal anaesthesia is not necessary. Animals reliably wake up approximately 10 min after the end of narcosis.

## 6.2 Donor operation and preparation *ex situ*

The mouse intended for donor operation receives narcosis in the above mentioned way. Afterwards, the mouse which lies on its back gets fixed with tape at its limbs to a corkboard and Isoflurane is applied via a tube to the mouse's nose. Hereby, interim awakening of the animal can be avoided.

The operation starts with median laparotomy. The abdomen is kept open by use of two needles, fixing the peritoneum to the corkboard. The bowel gets enveloped into a moist compress and put away to the right side outside the mouse. Then the IVC and the abdominal aorta can be prepared with a cotton swab. Using 1 ml syringe and 25G needle, the Aorta is cannulated and as much blood as possible is aspirated. The initially performed abdominal cut to the lower margin of the left hepatic lobe has to be extended to the sternum. Next, two more cuts have to be done along the costal arches to enable separation of the diaphragm from its costal and sternal adherence. In the following step the thorax is opened by cutting along the midaxillary line right up to the upper thorax aperture. Fixation of the sternum above the mouse's head (e.g. with a needle holder) guarantees free access to the opened thoracic cavity. Via puncture of the IVC the heart can now be flushed with cardioplegic fluid and it stops beating. After preparation and resection of the thymus gland, the inferior vena cava and the superior vena cava (SVC) are ligated with 6/0 silk. Silk ties are placed around the right and left pulmonary vessels (RPV / LPV) to exclude the lungs. Finally, the azygos vein (AV) is ligated. The donor heart is gently detached from the surrounding tissue with blunt dissection. For anastomosis of the recipient's abdominal aorta (AbdA) the donor's Aorta ascendens (AscA) and pulmonary artery (PA) remain open (see *fig. 2*). By now the heart can be harvested by sharply dividing along the esophagus and cutting the ligated vessels. The ascending aorta is cut below the brachiocephalic artery and the main pulmonary artery is cut proximal to its bifurcation.

Preparation of the pulmonary trunk and the Pars ascendens aortae has to be performed *ex situ*. Both of them are piggybacked on a curved pair of pincers and cut with a micro-surgical pair of scissors. Hereby you get two homogeneous and relatively long vessel stumps that enable easy implantation during the recipient operation. Until this moment the cardiac graft has to be stored in 4°C cold cardioplegic solution (e.g. Bretschneider's cardioplegic solution, Köhler Chemie, Alsbach, Germany).

## 6.3 Recipient preparation and transplantation

For the recipient operation narcosis, fixation on the corkboard, and median laparotomy from the symphysis to the lower margin of the left hepatic lobe are performed as described above during the donor operation. After opening the abdominal cavity and fixation of the peritoneum with two needles (alternatively a self-retaining retractor can be used) the bowel

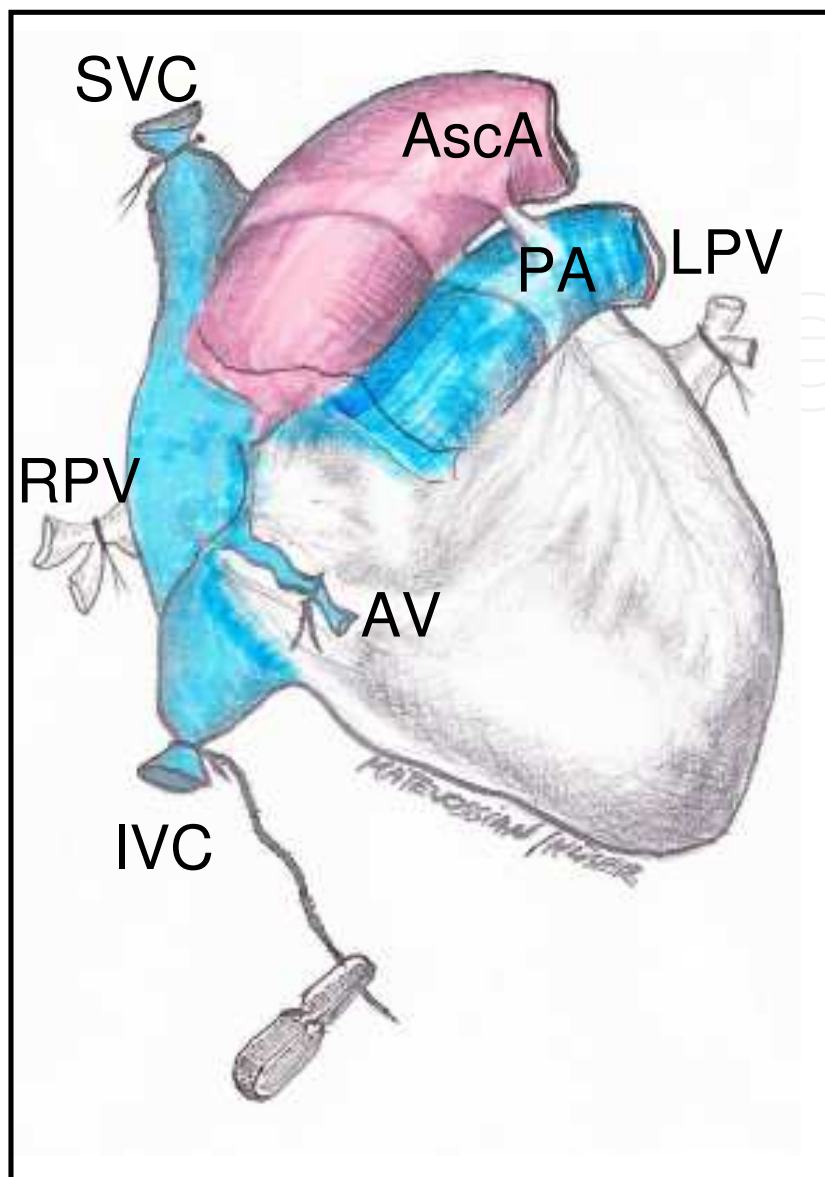


Fig. 2. Depiction of vascular ligatures at the donor's heart for organ harvesting

is enveloped into a moist compress and put away to the right side outside the mouse. Repetitive soaking of the compress with sterile saline guarantees a humid environment for the bowel during the entire procedure. The retroperitoneal space can be opened easily by gentle rotary motions with two cotton swabs. An additional cotton swab fixed in a needle holder can be used for permanent dislocation of the sigma and the left kidney outside the operating area after dividing the meso-sigmoid. Now the large vessels - Aorta abdominalis and IVC - are accessible and have to be separated from surrounding fatty tissue and adjacent lymph nodes. The preparation of the vessels starts directly below the outlet of the renal arteries and has to be performed to the aortic bifurcation. In case of lumbar veins in this segment, they have to be exposed and coagulated to assure that both the aorta and the IVC lie unfettered. Two vessel clamps have to be positioned at the ends of the prepared segment, respectively, to interrupt the flow in both the aorta and the IVC. The distal clamp has to be put first, to assure partially filled vessels for aortotomy and venotomy in the next step (see *fig.3*).



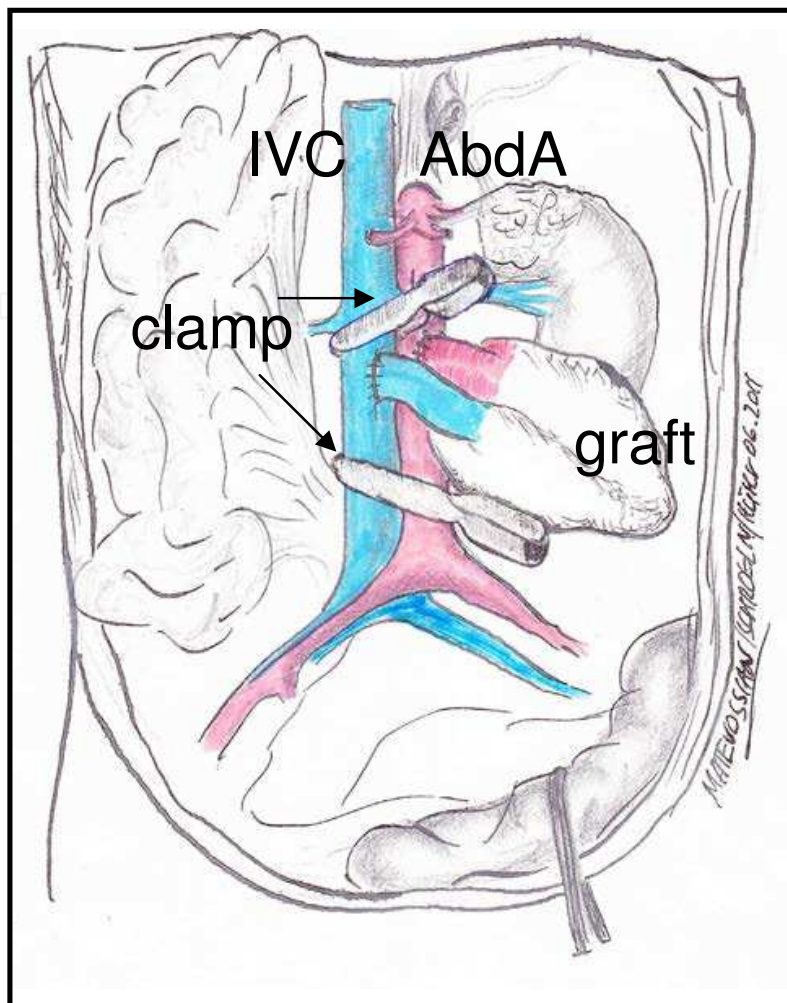


Fig. 3. Recipient preparation

After opening of the retroperitoneal space and positioning of the bowel which is enveloped into a moist compress and put away to the right side outside the mouse the colon sigmoideum and the left kidney are pulled away with a cotton swab fixed in a needle holder to guarantee free access to the Aorta abdominalis (AbdA) and the vena cava inferior (IVC). Two vascular clamps are positioned first just below the renal vessels and second at the proximal side of the bifurcation.

In the following step, the aorta has to be opened by a short longitudinal section using micro-scissors. This can be done easily without prior arteriotomy by a 25 or 30 gauge needle and is extended to a length of equal to the donor's ascending aorta. After flushing the vascular lumen with saline, an end-to-side anastomosis of the pars ascendens aortae of the cardiac graft and the abdominal aorta has to be performed. The silk thread that has been left long at the IVC during the donor operation can now be inserted in a small clamp and therefore helps to move the heart without any injuring touch into the right position in relation to the recipient's vessels. Two stitches in the edges with a 10/0 monofil thread fix together the donor's and the recipient's aorta. The graft is shifted to the right inferior abdomen of the mouse to facilitate free access to the left lateral side of the aorta. Now the first continuous suture can be performed by means of 4 stitches. For the knot we use the thread of the opposite edge stitch (see *figure 4a*). At this time point the donor heart is flipped from the right side of the mouse to the left using the long thread at the IVC. The corkboard can now

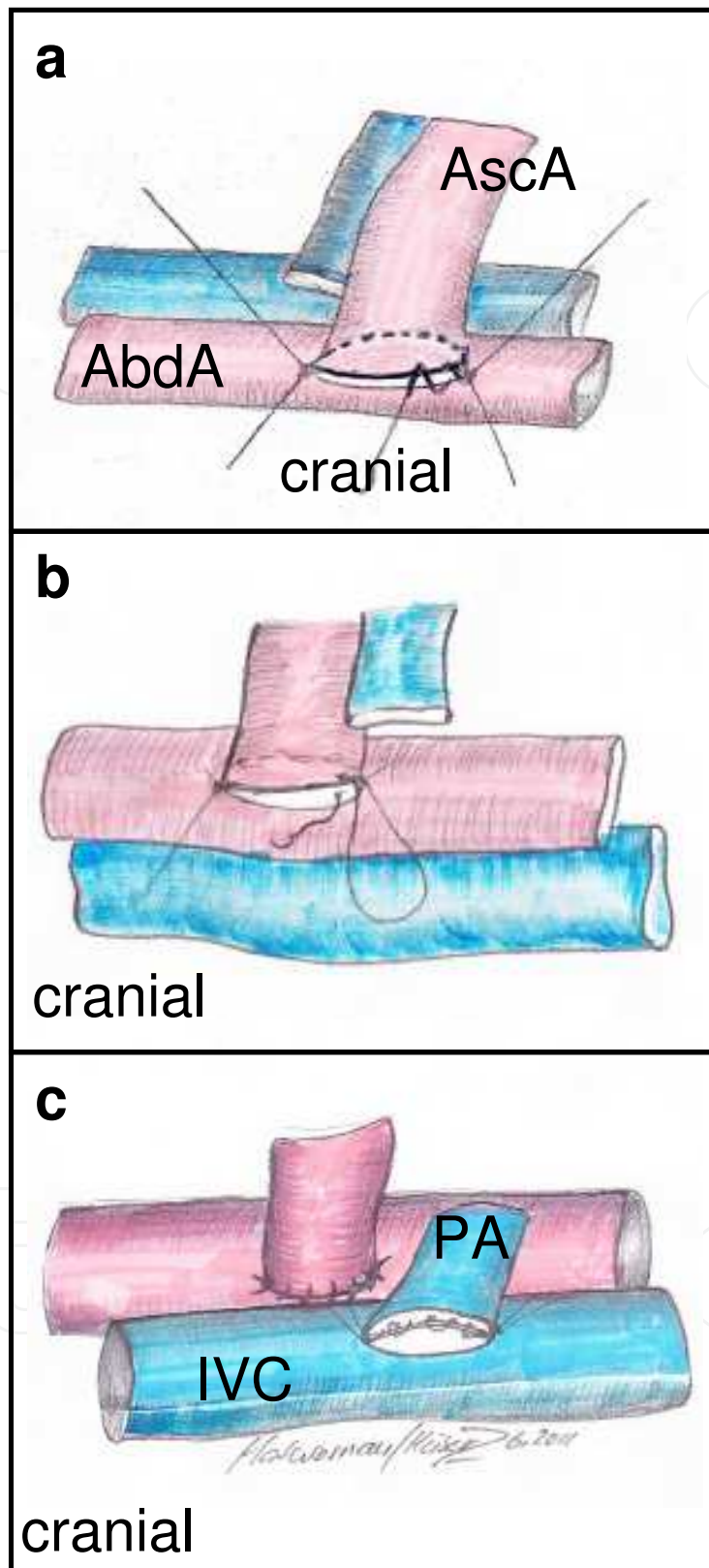


Fig. 4. Implantation of the vascularized graft  
a) After flushing the vascular lumen with saline, the end-to-end anastomosis of the pars ascendens aortae of the cardiac graft (AscA) and the recipient's abdominal aorta (AbdA) is performed continuously with 4 stitches (direction of stitches: outside-inside / inside-

outside). Two edge stitches with a 10/0 monofil thread fix the donor's and the recipient's aorta. Gently tension to a thread of the ligature at the IVC of the graft enables easy positioning of the cardiac graft with regard to the recipients vessels.

b) The corkboard is turned and now the arterial "back-wall" becomes the "front-wall" which can be sutured continuously with 4 stitches (direction of stitches: outside-inside / inside-outside). The knot is done with the initially performed edge stitch and cut.

c) After venotomy again 2 edge stitches are necessary to adapt the pulmonary artery (PA) to the inferior vena cava (IVC). It is important to fix the "back wall" with slightly more tension compared to the "front wall" to obtain best possible exposition of the operation area. The first continuous suture is now performed inside the lumen (4 stitches, direction of stitches: inside-outside / outside-inside). Therefore the first stitch has to be performed from the outside to the inside and at the end of the suture the thread has to be stitched outside before knotting outside the vein's lumen. The last suture then can be performed continuously outside the vessel with 5 stitches at the "front wall" (direction of stitches: outside-inside / inside-outside).

be half-turned, so the vessel's "back wall" becomes "front wall" and the suture is performed accordingly with 4 stitches (see *figure 4b*). Finally, after knotting, the threads have to be cut to a length of approximately 2 mm. The next step is the venotomy. The incision with the micro scissors is performed just as the aortic incision and proportionately to the lumen of the pulmonary trunk of the donor. Again two edge stitches have to be done. Hereby it is important to fix the "back wall" with slightly more tension compared to the "front wall" to obtain best possible exposition of the operation area. This is due to the fact, that the end-to-side anastomosis of the opened IVC and the pulmonary artery of the donor has to be closed with a running suture in the inside of the IVC. For this purpose the suture must be stitched from the outside to the inside of the vessel lumen at the beginning and vice versa at the end of the suture before the knot can be done outside the lumen. For the continuous suture again 4 stitches are necessary (see *figure 4c*.)

After the knot with the proximal suture, the right wall of the IVC and the pulmonary artery of the donor are closed with another 5 stitches of a continuous suture outside the IVC. After knotting with the thread from the distal edge stitch both threads are cut to a length of approximately 2mm. Finally, the heart can be reperfused by opening the clamps after a cold ischemia period of approximately 25 minutes. The distal clamp is removed first to check the anastomosis of the IVC. Rapid stain towards bright red, refilling of the coronary vessels, and time-displaced beginning of cardiac contractions indicate the successful performance of the operation. After cleaning the heart from residual adjacent tissue particles and repositioning of the bowel into the abdominal cavity, the abdomen is closed by means of a continuous suture of the musculature and the peritoneum, respectively, and completing closure of the skin with single stitches (all using 5/0 monofil non-resorbable thread). Then the Isoflurane leading tube is removed from the animal's head. Within the next minutes the mouse awakes from narcosis. The recipient's operation takes approximately 35 minutes. The cage is put under a heating lamp for another 20 minutes and the recipient gets tramadol drops for post-operative analgesia.

## **7. Potential of heterotopic heart transplantation in mice for elucidating molecular mechanisms of graft rejection**

Graduation of rejection after solid organ transplantation is primarily based on histopathological changes. Detection of immunological effector mechanisms display only a

minor role in this field and cannot necessarily be correlated to the severity of graft rejection. Hyperacute, acute, and chronic rejections can be discriminated, but the impression must not be created that they are chronologically consecutive phases of rejection. Whereas hyperacute rejection emerges quite rapidly after transplantation, it is entirely possible, that acute and chronic rejection occur simultaneously. Therefore, each rejection episode is designated to a macroscopic and a histopathological image rather than to a concrete point in time (see also *fig.5*).

The molecular and cellular mechanisms of the innate and the adaptive immune system responsible for acute and chronic graft rejection have been studied in the experimental setting of heterotopic cardiac transplantation in detail. In vertebrates, innate immunity mediates early host defence and comprises inflammatory cells that express receptors detecting conserved pathogen associated molecular patterns (PAMPs). Beside non-cellular mediators capable of microbial recognition (e.g. the complement system) especially natural killer (NK) cells constantly participate in surveillance for intact self by sensing the presence of autologous major histocompatibility complex molecules. In this context we could demonstrate the importance of NK cells during rejection of cardiac transplants. Acute graft rejection includes massive NK and NKT cell infiltration into the transplanted organ. We found that NK and NKT cells, contribute to the activation of alloreactive T cells. Furthermore, inhibition of NK and/or NKT cells in absence of the co-stimulatory receptor CD28 induced long-term acceptance of semi-allogeneic grafts transplanted to CD28-deficient recipients [Maier et al., 2001]. In the same situation, fully allogeneic grafts were rejected in CD28 deficient hosts, although at later time point compared to wild type controls. These findings clearly point out the advantage of the *in vivo* model of cardiac transplantation allowing to create an either completely or semi-allogeneic constellation between donor and recipient. Importantly, the generation of allogeneic response against grafts involves numerous activation pathways and the impact of single components is often not visible unless this complexity is reduced. Moreover, NK1.1 positive (NK/NKT) cells could not only be assigned to have relevant influence on rejection of cardiac grafts, these data also hint to the important and close interaction between the innate and adaptive immune system.

The advances in immunosuppressive therapy have made severe acute cellular rejection of organ grafts uncommon. This has revealed to the form of acute antibody-mediated rejection – now widely accepted as a distinct clinicopathologic entity [Colvin, 2007] – that is resistant to current immunosuppression and acts through graft rejection by activation of complement, vascular endothelial and smooth muscle cells, and by activation of macrophages, neutrophils or NK cells, respectively [Wehner et al., 2009]. More recent data have highlighted antibody-mediated rejection as a cause for chronic rejection [Colvin, 2007]. A detailed knowledge of the underlying mechanism could provide insights for effective therapeutic interventions, e.g. monoclonal antibodies to C5 [Wang et al., 2007]. However, the use of the murine heart transplant model to determine whether antibodies contribute to the rejection process had only limited success so far [Wehner et al., 2009]. Anatomical differences of the cardiac vascular bed between humans and mice might be a limiting factor in addressing this particular question. In contrast to mice, human coronaries contain vasa vasorum, and coronaries of the murine heart do not pass the surface but rather directly enter the myocardium after their origin. Thus, investigating humoral rejection processes, the model of heterotopic heart transplantation in mice is only applicable to a limited range.



The adaptive immune response as an essential element of the rejection process is mainly characterized by an engagement of T cells. Genetically non-identical transplanted organs display peptides recognized as foreign to host T cells. Helper T cells of the recipient become activated by foreign peptides that are displayed on antigen presenting cells (APC) in the donor organ. Interaction of activated helper T cells with B cells results in production of antibodies against the graft and activation of other immune cells, e.g. macrophages. In addition, allogen-specific CD8 cytotoxic T cells are known to be important for promoting transplant rejection in humans. This is in line with events during heterotopic allograft rejection in mice where we and others found a predominant activation of CD8 T cells in the transplanted heart of recipients [Hüser et al., 2010; Schnickel et al., 2004]. *In vitro* both, CD4 and CD8 T cells, proliferate in response to allogeneic stimuli. However, we could not detect activated CD4 T cells within cardiac grafts. In contrast, CD8 T cells were recruited in high numbers into the allograft and showed a phenotype of activated effector T cells [Hüser et al., 2010]. Again this indicates that the *in vivo* model of heterotopic heart transplantation in mice has great over *in vitro* studies and closely resembles the events taking place in humans.

Both, B and T lymphocytes use antigen receptor engagement to initiate distinct signal transduction pathways that affect cellular responses. It is well established that adaptor molecules regulate signaling of receptor proximal events inducing gene expression or cytoskeletal rearrangement. Beer et al. identified the putative adaptor protein SLY1, that is preferentially expressed in T and B lymphocytes and is defined as target for antigen receptor signal transduction with an important role in the adaptive immunity. It was shown that SLY mutant mice reveal impaired lymphoid organ development and antigen receptor mediated lymphocyte activation. Using the model of heterotopic cardiac transplantation, we were able to show extended allograft survival. Thus, signaling events mediated by SLY1 protein in cells of the adaptive immune system appear to be of importance for mechanisms inducing transplant rejection [Beer et al., 2005].

Chemokine receptors and their ligands, expressed by responding leukocytes and the inflamed transplant tissue, are responsible for the recruitment of alloreactive immunocytes into the graft. According to Hancock, several important points have emerged so far from murine *in vivo* transplant studies. (1) Targeting a single chemokine is in most cases ineffective in prolonging allograft survival, (2) chemokine receptors differ in their importance as targets in alloresponses, and (3) effects of concomitant immunosuppression can modulate the outcome of chemokine receptor targeting in otherwise untreated mice [Hancock, 2002]. The innate and adaptive immune reactions are initiated before, at the time of transplantation (e.g. by ischemia-reperfusion injury), and after transplantation by non-immunological and immunological factors, leading to graft vasculopathy with a diffuse narrowing of the coronary arteries and an adventitial fibrosis as common signs of chronic graft rejection. Chronic rejection is the response of the recipient's organism towards the cumulative injury to the transplanted graft over time, with involvement of cellular and humoral (antibody mediated) components [Chapman et al., 2005]. Best established model of chronic rejection in the mouse is the heterotopic cardiac allograft [Cornell et al., 2008]. On the basis of this *in vivo* model we could characterize the role of chemokine receptor CCR4 in chronic transplant failure. In accordance with Hancock [Hancock, 2002], absence of CCR4 receptor *per se* is not sufficient to achieve long-term transplant survival of fully allogeneic



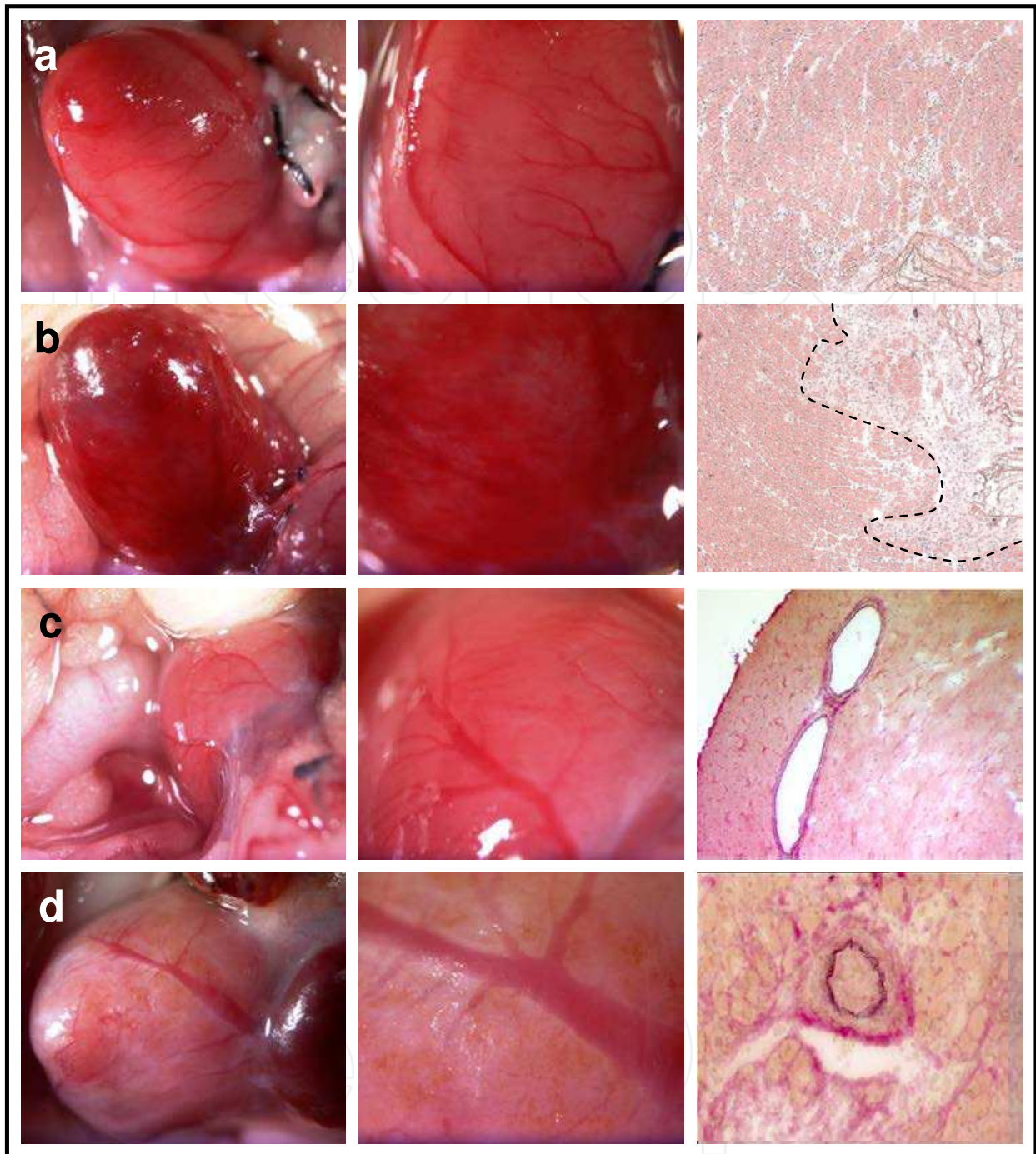


Fig. 5. Macro- and microscopy of transplanted hearts at different time points  
a) syngeneic hearts 7 days post transplant and tissue section stained with haematoxylin and eosin, showing no signs of rejection, whereas in b) allografts 7 days post transplant display myocyte destruction by invading mononuclear cells, interstitial edema and necrosis (inflammatory lesion is highlighted within the dashed line), and macroscopically massive haemorrhage lesions, c) syngeneic grafts 100 days post transplant with slight adhesion to the surrounding tissue but microscopically normal vascular anatomy and d) chronic rejection in allografts with exhibition of transplant vasculopathy caused by excessive hyperplasia in the intima 100 days post transplant; a-b) HE staining at magnification x40, inflammatory lesion is highlighted in b, c-d) van Gieson staining at magnification x60.

donor hearts in CCR4-deficient recipients and only marginal prolongation of heart survival was noticed. In addition, no significant immunohistological difference in cellular infiltration comparing wild type and CCR4-deficient mice could be observed [Hüser et al., 2005]. In contrast, injection of Gallium nitrate, known to delay graft rejection, resulted in a significantly prolonged persistence of heart action in CCR4-deficient mice compared to wild type controls [Hüser et al., 2005]. These results could be confirmed by findings in CCL17-deficient mice, CCL17 being the specific ligand of CCR4 [Alferink et al., 2003]. Besides constitutively expressed chemokines like CCL19 and CCL21 that mainly recruit naive T cells in the lymph node, inflammatory chemokines like CCL2, CCL3, CCL17 and CCL22 mainly cause accumulation of activated T cells and T memory cells in inflamed organs. Inhibition of several of these chemokines have been shown to be beneficial for graft survival [Tan et al., 2005].

Recruitment of activated lymphocytes into inflamed tissue is of major importance for the control of immune responses. Beside chemokines, the role of the leukocyte integrins in the immunological reactions of organ transplant rejection is essential. The integrin LFA-1 is expressed on all leukocytes as an adhesion molecule and has a significant role not only in lymphocyte migration but also in mediating T cell interaction with APC and consequently T cell priming [Hogg et al., 2003]. Function of LFA-1 is tightly controlled by regulating its activity state [Evans et al., 2009]. Generating a mouse mutant that expresses constitutively active LFA-1 (LFA-1<sup>d/d</sup>) [Semmrich et al., 2005] we could point out for the first time *in vivo* the importance of integrin deactivation on immune response inducing allograft rejection [Hüser et al., 2010]. We demonstrated that regulating LFA-1 activity from an active to an inactive state promotes successful activation, clonal expansion, and generation of effector T cells in response to allogeneic stimuli *in vivo*. Defective LFA-1 deactivation furthermore negatively affects recruitment of all major leukocytes from the innate and the adaptive immune system known to be involved in transplant rejection. By using the model of heterotopic cardiac transplantation we were able to provide direct *in vivo* evidence that regulating LFA-1 deactivation might be as important as regulating LFA-1 activation for effective immune responses during allograft rejection.

Those examples for studies in gene knock-out and transgenic mice using organ allografts identified the central role of a variety of target cells, chemotactic mediators, signalling components, and adhesion molecules on lymphocytes. As such, the described model of heterotopic cardiac transplantation in mice can be considered as a valid animal model and serves as an important achievement for clinical development of strategies that interfere with immune responses inducing transplant rejection.

## 8. Conclusion

Advances in the field of immunosuppression and better understanding of immunological courses in the last years contributed to establish organ transplantation as regularly performed clinical therapy and helped patients with severe renal, hepatic, pulmonary, and cardiac diseases to achieve longer survival and better quality of life. Short-term outcome after organ transplantation is impressive, whereas long-term graft survival remains to display a crucial problem. Induction of donor-specific tolerance towards transplanted tissue remains to be the aim.

Heterotopic heart transplantation in mice is a vascularized immunological model and the recipient is not dependent on maintenance of circulation and cardiac functioning. As an *in vivo* model it has been shown to be an ideal compromise between clinical reality and experimental reproducibility of underlying essentials that peak in acute rejection or chronic transplant failure over time, however immunological differences between mice and human beings must be kept in mind. And, if so, mice as an *in vivo* model of first choice will continue to push forward our immunological understanding of immune reactions in transplantation.

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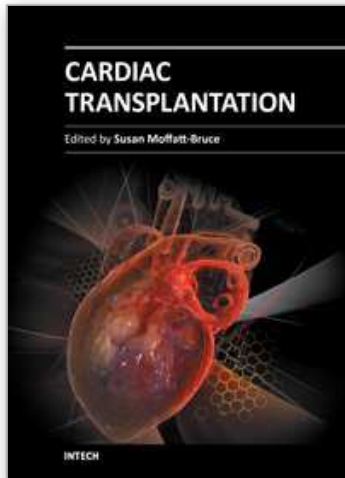
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We are truly in an era of change not only in terms of technology but in the type of patient we are caring for. That is why I feel this book is exciting in that it presents the team approach to the transplant patient. I am confident that the pioneers of cardiac transplantation would be pleased with our response to challenges in healthcare today and be pleased with the final product.

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