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Comparative Study of Skin Graft Tolerance and Rejection in the Frog *Xenopus Laevis*

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1. Introduction

Over the last few decades, the amphibian *Xenopus laevis* has been used as a unique comparative model to study the developmental and immunological aspects of tissue transplantation as well as self tolerance (Du Pasquier, Schwager et al. 1989; Robert and Ohta 2009). The immune system of *X. laevis*, one of the best defined outside mammals and chickens, is fundamentally similar to that of mammals. Notably, the evolutionary distance of *X. laevis* from mammals permits distinguishing species-specific adaptations from more conserved features of the immune system. In addition, advanced genetic resources including the full genome sequence of the *X. leavis* sister species *X. tropicalis*, a large collection of EST, cDNA and genomic (BAC, Fosmid) libraries for both species, efficient transgenesis and genome wide mutagenesis has markedly empowered *X. laevis* as a biomedical model. Furthermore, several different major histocompatibility complex (MHC) defined inbred strains of *X. laevis*, as well as clones sharing identical MHC haplotypes but differing at multiple minor histocompatibility (H) loci, provide a unique opportunity to study T cell regulation *in vivo* by skin graft. More information about these resources can be found on the Xenbase website (http://www.xenbase.org/common/) (Bowes, Snyder et al. 2009) and the *X. laevis* research resource for immunobiology (http://www.urmc.rochester.edu/mbi/resources/Xenopus/) (Robert 2006).

The objectives of this review are first to provide short background information on the immune system and skin graft biology in *X. laevis*, and then to examine the use of the minor H-Antigen (Ag)-disparate skin grafting model system in *X. laevis* isogenic clones and to investigate *in vivo* the immunostimulatory properties of certain heat shock proteins (hsp) such as gp96 and hsp70 with particular emphasis on minor H-Ag specific T cell responses. We will also consider the possible role of nonclassical MHC class Ib molecules in this context. Furthermore, we will re-evaluate old data on induction of long term immunologival memory and immune tolerance in *X. laevis* larvae to skin graft Ags in the context of immune regulation. Finally, we will discuss the possibility to use hspS and new genetic tools.

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1 All animals were handled under strict laboratory and UCAR regulations (Approval number 100577 / 2003-151), minimizing discomfort at all times.
(genomic and transgenic technology) to revisit this immune regulation to skin Ags during development of *X. laevis*. It is our conviction that by integrating the unique biological features of *X. laevis* with the recently advanced genetic resources of this comparative model, it will be possible to answer critical questions about the development of self-tolerance as well as autoimmunity.

### 2. The immune system of *Xenopus laevis*

The frog *X. laevis* has been used to study skin graft rejection as well as tolerance for the last few decades. The immune system of *X. laevis*, a genus whose common ancestors with mammals diverged 350 million years ago (Kobel and Du Pasquier 1975; Evans 2008), is fundamentally similar to that of mammals. Importantly, this extensive evolutionary distance allows one to distinguish species-specific adaptation from conserved features of the immune system. The *X. laevis* immune system is characterized by T and B lymphocytes with RAG-mediated rearranging TCR and Ig genes, MHC class I- and class II-restricted T cell recognition (Du Pasquier, Schwager et al. 1989; Robert and Ohta 2009), as well as innate immune cells such as macrophages and NK cell (Horton, Horton et al. 1998; Horton, Minter et al. 2000; Robert, Ramanayake et al. 2008; Morales, Abramowitz et al. 2010). Interestingly, *X. laevis* lacks lymph nodes but it does have both a thymus and a spleen similarly to mammals.

The additional developmental transition occurring during metamorphosis in *X. laevis* results in two different life stages, the larvae and the adult, which provides a unique opportunity of working with two distinct immune systems. Unlike mammals, larvae have external development; therefore, they are amenable to experimental manipulation and there is easy accessibility of early developmental stages free of maternal influences. For example, since large areas of the larvae are transparent, the thymus is easily distinguishable and it is also relatively simple to perform thymectomy on these animals which will render them free of T cells in their larval as well as adult life. During metamorphosis the immune system undergoes a remarkable developmental transformation during which surface major histocompatibility complex (MHC) class Ia (class Ia) expression becomes detectable for the first time on erythrocytes and splenic leukocytes (Flajnik, Kaufman et al. 1986; Flajnik and Du Pasquier 1988; Rollins-Smith, Flajnik et al. 1994). Interestingly, NK cells that are not detected during larval life emerge concurrently with class Ia expression (Horton, Stewart et al. 2003). *X. laevis* larvae are different from adults since they are naturally class Ia deficient, but importantly these animals are immunocompetent and they have thymus-dependent CD8 T cells (Flajnik, Kaufman et al. 1986). In addition, certain nonclassical MHC class Ib (class Ib) genes have been found to be expressed in the thymic anlage very early in ontogeny and preferentially by thymocytes (Goyos, Ohta et al. 2009; Goyos, Sowa et al. 2011). The implication of this on the T cell repertoire of early larval stages is under investigation. MHC class II antigen expression during larval life is restricted to the thymic epithelium centrally, and to B lymphocytes and accessory cells in the periphery: whereas, it is constitutively expressed on virtually all thymocytes and mature peripheral T as well as B cells in adults (Flajnik, Kaufman et al. 1986; Du Pasquier and Flajnik 1990; Flajnik, Ferrone et al. 1990). Thus, the larval and adult *X. laevis* immune systems have critical differences as well as similarities. As such, comparisons between these two developmental stages afford unique opportunities to investigate in vivo developmental and immunological aspects of tissue transplantation. Also the differential expression of class Ia together with the ease of larval
Experimental manipulation allows us to explore questions regarding MHC-restriction, autoimmunity, and the development of self-tolerance that can not be easily studied in other animal models.

A major attribute of the *X. laevis* model is the availability of different MHC-defined strains and clones. In addition to the MHC homozygous inbred strains, the J and F strains (Du Pasquier and Chardonnens 1975; Tochinai and Katagiri 1975), the *X. laevis* model also includes MHC-defined isogenic clones of frogs, such as the LG-15 and LG-6 that share the same heterozygous MHC haplotypes (a/c) but differ at multiple minor histocompatibility (H) loci (Kobel and Du Pasquier 1975; Kobel and Du Pasquier 1977). These clones are generated by gynogenesis, a procedure where diploid eggs from *X. laevis/X. gilli* hybrids are only activated by UV irradiated sperm, and thus the spermatozoid DNA does not contribute any genetic material to the offspring. This system allows us to easily pool cells from different frogs and perform adoptive cell transfers since the clones share identical genetic make up (Maniero and Robert 2004). In addition, we also have available a transplantable thymic lymphoid tumor named 15/0 (originally a spontaneously growing tumor derived from a LG-15 clone) that is tumorigenic in both LG-15 and LG-6 clones (Robert, Guiet et al. 1994; Robert, Guiet et al. 1995; Robert and Cohen 1998). Interestingly this tumor does not have class Ia protein expression however it does express several class Ib molecules (Robert, Guiet et al. 1994; Salter-Cid, Nonaka et al. 1998; Rau, Cohen et al. 2001). As a result, the accessibility of different animals and reagents has provided us the unique opportunity to study T cell regulation *in vivo* by skin transplantation.

3. Skin graft rejection in adult *Xenopus laevis*

Skin graft rejection is a well-established technique in *X. laevis* that has been used for determining the segregation of both major and minor H-Ag loci (Chardonnens and Du Pasquier 1973; Du Pasquier and Bernard 1980; Ramanayake, Simon et al. 2007).

Fig. 1. Skin graft rejection in *Xenopus laevis*. (A) Allograft one day post transplantation, 0% rejection. The entire graft appears silvery due to the presence of irridophores. In (B) and (C) the grafts are undergoing rejection 50 and 90% respectively. (*) indicates areas of the graft that are still surviving. The rejected areas of the grafts are highly vascularized. (D) Representative image of a fully (100%) rejected skin graft. This graft is “dull” and does not have any more surviving irridophores.

We have recently described this technique in details (Nedelkovska, Cruz-Luna et al. 2010). Succinctly, *X. laevis* adults are skin grafted by cutting a 5mm\(^2\) piece of donor ventral skin.
Skin Grafts – Indications, Applications and Current Research

(abdominal skin which appears silvery due to the presence of irridophores) and inserting it under the dorsal skin of the recipient (Chardonnens and Du Pasquier 1973; Nedelkovska, Cruz-Luna et al. 2010). It is critical to handle the graft gently and avoid damage with the forceps, which would obscure the results. Also care must be taken to avoid introducing large air bubbles under the skin because that can cause displacement or loss of the graft. After 24 hours, a window of overlaying host skin covering the graft is removed and then the graft can be easily visualized using a dissecting microscope. Skin graft rejection is then monitored by determining the percent of irridophore destruction over time. Rejection is considered complete when the entire exposed graft has become “dull” and all the irridophores have been entirely destroyed (Figure 1).

3.1 Conserved role of T cells in skin graft rejection

In adult *X. laevis*, as in mammals, the kinetics of skin graft rejection are dependent on the number of MHC mismatches as well as minor H-Ags between the donor and the recipient frogs. If the graft is either an autograft, syngenic or an isograft, it will never be rejected by the host. However, if the donor skin displays one or two MHC haplotype mismatches, then there will be an acute skin graft rejection that will take 18 to 22 days at 21°C to complete (Table 1). Furthermore, if the frogs share the same MHC haplotype but only differ by minor H-Ags then the graft will undergo chronic rejection, which will take between 30 to more than 100 days at 21°C for full rejection depending of the genetic combinations (Table 1). With *X. laevis* being ectothermic, temperature has a profound effect on skin graft rejection. For instance, at 27°C minor H-Ag-disparate grafts between individuals of the partially inbred F strain are rejected in 23-37 days, which is significantly faster than the rejection at 21°C that takes 60-100 days (Robert, Guiet et al. 1995). *In vitro*, *X. laevis* immune functions are also affected by temperature (Hsu 1998). For example, *in vitro* T-cell proliferation in MLR (Mixed Lymphocyte Reaction) or induced by mitogen (Meier 2003) and proliferation of lymphoid thymic tumor cell lines (Robert, Guiet et al. 1994), occurs faster at 27°C (optimum) than at lower temperature (18–25°C). Therefore, temperature has to be taken in account when comparing *in vivo* and *in vitro* responses against alloantigens.

The strict T cell dependency of skin rejection has been clearly established in *X. laevis* by thymectomy at early developmental stages before the full differentiation of T cell precursors (5-6 days post-fertilization; st. 47/48 (Horton, Horton et al. 1998) stages based on (Nieuwkoop and Faber 1967)). Adult frogs that have been thymectomized at early developmental stage fail to reject both MHC as well as minor H-Ag-disparate allografts (Barlow and Cohen 1983; Arnall and Horton 1987; Robert, Guiet et al. 1997). In contrast, adults that have been thymectomized either during mid larval (st. 53-55) or late larval (st. 56-58) development remain capable to reject MHC-disparate grafts. This most likely is due to the fact that mature T cells are able to migrate out of the thymus before it is removed; therefore, they can have effector functions in the periphery. These data demonstrated that skin graft rejection in adult *X. laevis* is thymus dependent and suggested that like in mammals allograft rejection is mediated by CD8 as well as CD4 T cells. Additionally, there is immunological memory against minor H-Ag because second set skin grafts are rejected in an accelerated fashion while third party grafts are not, suggesting that these responses are specific to the immunizing alloantigens (Table 1) (Nagata and Cohen 1983).

In order to assess more directly the role that CD8 T cells play in these responses *in vivo*, depletion by antibody treatment was used. CD8 T cells were depleted using the AM22
mouse anti-X. laevis CD8 monoclonal antibody (Flajnik, Ferrone et al. 1990). AM22 is specific for CD8 T cells because cells stained with this antibody express a pan-T cell marker (XT-1) as well as high levels of CD5 and CD45 (Gravenor, Horton et al. 1995; Robert, Sung et al. 2001). Moreover, cells stained by AM22 are not detected in frogs that are thymectomized during early larval life and lack T cells (Gravenor, Horton et al. 1995; Robert, Guiet et al. 1997). AM22 depletion experiments showed that in the absence of CD8 T cells MHC-disparate allografts did not undergo the typical acute rejection pattern but underwent slower rejection that took 7 days longer to complete (Rau, Cohen et al. 2001). Interestingly, these grafts were indeed completely rejected even if more mAb AM22 injections were used to prolong the CD8 T cell depletion effect. This suggests that other cells like CD4 T cells may also be involved in the response against skin Ags. To date this possibility has not been investigated due to the unavailability of antibodies against X. laevis CD4. However, recently our lab has generated and is currently characterizing a single chain CD4 antibody which will allow exploration of the importance of CD4 T cells in allograft rejection.

To further characterize the CD8 T cells involved in graft rejection, our lab adapted a whole-mount immunohistology technique. This procedure allows us to visualize lymphocytic infiltration into unfixed transplanted skin tissues using fluorescent antibodies (Ramanayake, Simon et al. 2007). Additionally, this method preserves the tissue structure and we can use several antibodies conjugated to different fluorophores to see exactly where cells are located and distinguish what kind of cells are present in the grafts. Therefore, this technique is a powerful tool which we can use to characterize and monitor immune effector cells mediating the immune responses of X. laevis against skin rejection Ags.

Using whole-mount immunohistology we found that, unlike isograft controls, MHC-disparate grafts that were undergoing rejection were infiltrated with a large number of CD8 T cells. These CD8 T cells were mainly distributed in areas where the graft was not yet rejected and the silvery irridophores were still persisting. Moreover, there was an inverse correlation between the percent rejection and number of infiltrating cells. For example the most prominent CD8 T cell infiltration occurred at day 7 when there was only 50% rejection. Additionally, these grafts also had significant infiltration of class II positive cells which were more numerous than the CD8 T cells. As mentioned before all adult leukocytes have class II expression in adult X. laevis, therefore, the majority of the class II positive cells in MHC-disparate grafts were actually CD8 T cells (~80%). This was also seen by cell morphology since most of the cells were small round lymphocytes although this does not exclude CD4 T cells. Besides lymphocytes, other cells morphologically similar to macrophages and dendritic cells (such as Langerhan cells) were seen in both allografts and isografts. These cells were already present in the grafts before transplantation, which suggests that they could potentially serve as antigen presenting cells (APCs). However, more of these cells from the recipient animal can infiltrate the graft and then migrate out to the spleen to prime more CD8 T cells.

As previously discussed, the main difference between MHC-disparate and minor H-Ag-disparate grafts is the time it takes for complete graft rejection, which is either acute or chronic, respectively. Therefore, one might assume that this is due to the lesser number of infiltrating effector T cells since only minor H-Ags are involved in these responses. On the contrary, however, we found that minor H-Ag-disparate grafts were infiltrated by similar numbers of both CD8 and class II positive cells, but with delayed kinetics (Ramanayake, Simon et al. 2007). In these minor H-Ag-disparate allografts the peak of immune cell
infiltration was also observed when the graft was about 50% rejected which in this case occurred 15 days after transplantation, rather than 7 days as in the case of MHC mismatched grafts (Table 1).

Whole-mount immunohistology is a very powerful technique to study infiltration of immune cells; therefore, we are currently using different antibodies such as X. laevis CD4 and HAM56 (a human macrophage marker which cross-reacts with X. laevis macrophages) which will allow us to visualize both effector cells as well as APCs, respectively.

### 3.2 Characterization of the immunological properties of heat shock proteins (HSPs) using skin graft rejection

Our X. laevis skin grafting model has been instrumental to get better insight into the immunological properties of certain hsp such as gp96 and hsp70. Hsps are evolutionarily ancient and highly conserved intracellular molecular chaperones that help with intracellular transport, folding of newly synthesized proteins, and prevent protein aggregation. In addition, hsps have been implicated in a variety of innate as well as adaptive immune responses. Notably, hsps have the intrinsic property to carry exogenous antigenic peptides from the tissues which are purified from and interact with endocytic receptors expressed by APCs. Once the hsp-Ags complexes enter the APCs the peptides are shuttled into the MHC class Ia cross-presentation pathway where they are processed and presented by class Ia molecules to CD8 T cells. Therefore, hsps have the ability to elicit potent CD8 T cell responses against the chaperoned Ags. Gp96 and hsp70 bind Ags differently. While the peptide binding of gp96 is unclear, hsp70 has defined peptide binding which is ATP dependent (Blachere, Li et al. 1997). Peptides can be loaded in vitro onto hsp70 by simply adding ADP to the reaction, whereas ATP addition results in peptide disassociation. Hsp70 preferentially binds peptides that contain 4-5 hydrophobic residues flanked by two basic residues (Castellino, Boucher et al. 2000), but it has been shown that 30 amino acid long synthetic peptides can also be complexed (Calderwood, Theriault et al. 2005).

As mentioned, the LG-6 and LG-15 clones share the same MHC haplotypes but differ by minor H-Ags, and frogs primed with a first set of minor H-Ag-disparate skin graft reject a second set skin significantly faster. Furthermore, this accelerated rejection is thymus-dependent and Ag specific (e.g., a third party skin graft rejection is not accelerated). This system has revealed to be ideal in investigating whether the ability of hsps to generate CD8 T cell responses is conserved between mammals and amphibians.

We found that if we first immunize LG-6 clones with either gp96 or hsp70 purified from LG-15 liver (meaning that those hsps would carry LG-15 minor H-Ags) and then graft them with an LG-15 allograft, the graft undergoes accelerated rejection in comparison to control unimmunized animals or animals that were immunized with LG-6 derived hsps (carrying self-Ags) (Robert, Gantress et al. 2002). Additionally, syngenic grafts were never rejected regardless of the immunization status of the animal, which rules out possible Ag-independent pro-inflammatory effect induced by the hsps. Furthermore, these in vivo responses are specific to the Ags chaperoned by the hsps because if an LG-6 animal is immunized with LG-46 derived hsp and is grafted with both LG-15 as well as LG-46 skin (these three LG clones share the same MHC haplotype (a/c) but differ in multiple minor H-Ags) only the LG-46 graft will have accelerated rejection, while the LG-15 graft will undergo a typical chronic rejection (Table 1). Moreover, immunization with hsp70-peptide complexes results in accelerated skin graft rejection, while immunization with hsp70 free of Ags (i.e.,
Ags eluted by ATP-agarose chromatography) does not change the rejection kinetics in comparison to unimmunized animals. These data indeed show that both gp96 and hsp70 are able to generate potent specific T cell responses in vivo against minor H-alloantigens.

<table>
<thead>
<tr>
<th>Type of skin grafts*</th>
<th>Minor H-Ag disparate</th>
<th>1 MHC haplotype disparate</th>
<th>2 MHC haplotype disparate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rejection at 21°C</td>
<td>30 - 100</td>
<td>18 - 22</td>
<td>18 - 22</td>
</tr>
<tr>
<td>Peak CD8 T cell infiltration</td>
<td>15</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Rejection after priming with same first set graft</td>
<td>16 - 30</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Rejection after gp96 or hsp70 immunization</td>
<td>20 - 30</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table 1. Summary of allograft rejection and immunological memory in adult X. laevis. Data is represented in days. ND, Not done or data not found in literature.

In mammals it is known that immune responses generated by hsps are mediated by CD8 cytotoxic T lymphocytes (CTLs). Using an in vitro killing assay, our lab confirmed that as in mammals, immunization with gp96 and hsp70 results in the generation of anti-minor H-Ag CTLs. Specifically, CD8 T cells purified from LG-6 animals primed with LG-15 tissue-derived hsp70 were able to kill class Ia positive LG-15 lymphoblast but not class Ia positive LG-6 blast targets nor class Ia negative 15/0 tumor targets. In contrast, CD8 T cells from animals immunized with Ag free hsp70 or unimmunized animals did not kill any of the blast targets. The same results were obtained using gp96 and different combinations of cloned frogs. Besides showing that the ability of hsps to generate CTL responses is conserved in amphibians, this study has provided definitive evidence of MHC class Ia restriction and Ag specificity of cytotoxic CD8 T cell effectors in X. laevis.

To explore in vivo the anti-minor H-Ag CD8 T cell effector capacity generated by gp96, our lab adapted a carboxyfluorescein diacetate succinimidly ester (CFSE) proliferation assay for X. laevis (Robert, Gantress et al. 2004). CFSE is a fluorescent dye that is incorporated into viable cells and upon cell division the CFSE content gets diluted in half, meaning that each daughter cell will have half the CFSE fluorescent intensity in comparison to undivided cells. This technique allows us to label, follow and calculate the percent of dividing cells as well as to determine the number of divisions that occurred. Using this approach, splenocytes from LG-6 frogs immunized with LG-15 gp96 were first labeled with CFSE and then were adoptively transferred into naïve LG-6 recipients that were previously grafted with a LG-15 skin allograft or a LG-6 syngenic graft. These experiments showed that the transferred CFSE labeled splenocytes accumulated in the spleens of animals that carried minor H-Ag-disparate skin grafts, while no such accumulation was detected in animals with syngenic grafts (Maniero and Robert 2004). Furthermore, by flow cytometry staining it was shown that the majority of CFSE+ dividing cells in these animals were CD8 T cells, which underwent several rounds of proliferation (3-4 cycles). This proliferation is not as robust as the one seen in mammals where up to 8 divisions can be seen. This can be due to several reasons including the difference in temperature as mentioned earlier or the strength of the TCR signal. Currently we do not know the status of other subsets of gp96 primed T cells to
minor H-Ags; however, since we know that CD8 depletion alone is not enough to completely abrogate skin graft rejection, this would suggest that other immune effector cells are also involved.

To test the effector function of adoptively transferred CFSE+ cells we monitored skin graft rejection. We found that unimmunized frogs carrying a minor H-Ag-disparate graft had accelerated skin graft rejection which reached 45-90% by day 10 after adoptive transfer of splenocytes (Maniero and Robert 2004). On the other hand, animals that carried isogenic grafts did not show signs of rejection. These rejection kinetics are reminiscent of a secondary T cell mediated rejection that occurs with animals primed with gp96 carrying minor H-Ag complexes. This means that gp96 is able to prime CD8 effector T cells that when adoptively transferred can recognize minor H-Ags presented in vivo by an allograft and kill the transplanted skin.

### 3.3 Possible role of nonclassical MHC class Ib genes in skin transplantation

As discussed above, CD8 T cells are critically involved in skin graft rejection in X. laevis and these responses are MHC class Ia restricted. However, the involvement of nonclassical MHC class Ib (class Ib) molecules in allore cognition has not been addressed. Class Ib genes in comparison to class Ia are heterogeneous genes with a limited tissue distribution and low polymorphism (Hofstetter, Sullivan et al. 2011). Both class Ia and most class Ib molecules have similar structure and need to associate with β2-m in order to be presented at the cells surface (Goyos, Guselnikov et al. 2007). Class Ib molecules, unlike class Ia, usually present Ags of a more limited variability or PAMPs including peptides as well as lipids and glycolipids. In X. laevis there are as many as 20 X. laevis class Ib (XNC) genes divided into 11 subfamilies (Flajnik, Kasahara et al. 1993; Goyos, Ohta et al. 2009). We have demonstrated that X. laevis has the ability to generate unconventional class Ib restricted anti-15/0 CTLs after hsp immunization (Goyos, Cohen et al. 2004). There is a possibility that after allograft or hsp immunization a population of class Ib mediated T cell effectors can arise. These effector cells can be both CD8 positive as well as CD8 negative as in the case in human and mice. Interestingly, we recently found expression of the XNC11 gene, in the skin of adult frogs. The XNC11 gene has a very unique expression pattern; it is almost exclusively expressed at low levels in the thymus, and at high levels by several thymic lymphoid tumors including 15/0 (Goyos, Ohta et al. 2009). We now have evidence of faint but consistent expression of XNC11 in the skin. So far attempts to modify the expression pattern of XNC11 by any inflammatory stimuli such as LPS and heat killed bacteria or by viral infection has been unsuccessful. Currently, we are working under the hypothesis that XNC11 is mainly found in the skin macrophages, Langerhan cells or unconventional T cells. If this hypothesis is correct, it is possible that XNC11 can present Ags to effector cells during allograft recognition or is involved in the regulation of specialized or unconventional skin resident T cells. Our lab is in the process of generating monoclonal antibodies against several different class Ib molecules including XNC11. These tools will allow us to directly address if XNC11 or other class Ib genes are involved in immune responses against skin minor H-Ags.

### 4. Immune responses and tolerance to skin antigens during larval and metamorphic stages

Of particular relevance for the immunological aspects of tissue transplantation, is the fact that unlike mammals, the X. laevis immune system undergoes striking developmental remodeling.
twice during its life: first during embryogenesis, and then again during the transition from larva to adult. The thymus, first colonized by embryonic stem cells a few days after fertilization, undergoes a second wave of stem cell immigration after losing most of its lymphocytes during metamorphosis. The embryonic and larval periods of thymocyte differentiation take place in different environments since during metamorphosis the whole organism is remodeled and many new proteins are expressed that could be considered antigenic by the larval immune system. The emerging adult lymphocytes, therefore, are likely to be subjected to a new wave of negative selection by the adult "self," resulting in a new balance of self-tolerance. In addition, MHC class I and class II genes are differentially regulated during metamorphosis. Although, larvae like adults have CD8 T cells, there is no consistent expression of MHC class Ia until metamorphosis, especially in the thymus.

In this section we will discuss the ability of *X. laevis* larvae to become tolerant to skin alloantigen. The induction of alltolerance is a very complex process governed by several different variables; therefore some conflicting data have been reported concerning its ontogeny as will be discussed later in detail. One of the differences that may affect tolerance induction is technical variability. Skin grafting in larvae is similar to grafting in adults but with several important differences. A first notable difference is that due to its fragility larval skin does not support transplantation onto a larval hosts and immune rejection is difficult to distinguish from tissue degeneration (Horton, Horton et al. 1993). For this reason, the use of ventral adult, instead of larval skin, graft onto larval recipients has been and is still favored. The adult skin is introduced under the larval skin on the head between the ears of the recipient (Chardonnens and Du Pasquier 1973). Another difference is that compared to adults, larvae are transparent. The adult skin is usually firmly fixed one day after transplantation and the transparent host skin retracts from the graft therefore removing the overlaying skin is unnecessary (Figure 2). Graft rejection is scored similarly to adults where the percent of irridophore destruction is determined. Several factors have been shown to affect the outcome of adult skin transplantation in tadpoles, including the size of the transplanted skin, as well as the genetic background and the developmental stage. Changes in temperature do not affect the percent of skin graft rejection; however they do influence the kinetics of rejection at all developmental stages. At 21°C grafts differing by 1 MHC haplotype are rejected in 40-55 days in comparison to 25-35 days at 24°C (Cohen, DiMarzo et al. 1985). Furthermore, size of the graft is very important for tolerance induction and in general larger grafts have an increased chance to be tolerized. Experiments that use outbred animals, which are genetically heterogeneous, should be considered with caution when compared to those that use MHC defined frogs differing by minor H-Ag loci only or by minor H-Ag loci plus one or two MHC haplotypes.

Another possible source of conflicting results concerns development, especially metamorphosis, which may play the most important role in generating tolerance. Metamorphosis in frogs, both initiation and completion, is under the control of the thyroid hormone (Furlow and Neff 2006; Tata 2006). The thyroid hormone starts being produced around stage 50, peaks at stage 60, and the levels are back to normal at the end of metamorphosis (Figure 3). Also there are two different thyroid receptors- α and β. Thyroid receptor-α is expressed early on after hatching while β comes up concomitantly with the thyroid hormone (Figure 3). In addition, the action of the thyroid hormone during metamorphosis is local and not systemic. This means that in certain tissues the changes associated with metamorphosis may start earlier than in other tissues and this may directly impact the ontogeny of tolerance. The complexity of the metamorphic transition is likely to result in marked individual variation even in clonal animals, including the differences in
Fig. 2. Larval skin graft. A. Minor H-Ag-disparate adult skin graft inserted on the head region of a tadpole. B. Enlarged image of the graft shows the presence of silvery iridophores, 0% rejection.

*Figure adapted from (Du Pasquier, Schwager et al. 1989) and (Furlow and Neff 2006)

Fig. 3. Overview of the changes occurring during metamorphosis. The different developmental stages of *X. laevis* are illustrated including the period of metamorphosis along with the morphological criteria used. Additionally, the relative expression of the thyroid hormone as well as the thyroid hormone receptors TR-α and TR-β is depicted. Also, total thymocyte number, which significantly decreases during metamorphosis, is shown to correlate with impaired T cell function (MLR activity).
the immune system. This is indeed observed by the individual variation in morphological changes (e.g. there are easily several day differences in the time of complete tails loss in cloned progeny).

In order to address some discrepancies in the literature we will first discuss the induction of tolerance to minor H-Ag followed by MHC-disparate grafts. The mechanism(s) as well as possible effector cells involved in allotolerance will also be considered. Finally, we will provide some perspective on using new tools and methodologies to more conclusively answer questions associated with tolerance.

4.1 Immune response and allotolerance in *X. laevis* larvae to minor H skin antigens

*X. laevis* tadpoles develop allorecognition at stage 49 (12 days post fertilization) which is accompanied by lymphocytic infiltration of the grafts (Horton 1969). In *X. laevis* there are three distinct periods throughout development during which there is a difference in the immune responses against skin allografts (Chardonnens and Du Pasquier 1973). Those include the periods before, during and after metamorphosis. Sibling studies showed that at stage 53 (period before metamorphosis or premetamorphosis) allografts from siblings were completely rejected (Table 2). Furthermore, larvae at the same stage were also grafted with allografts from unrelated donors and again the majority of grafts underwent complete rejection. Moreover, these tadpoles primed by a first skin graft, rejected a second set skin grafted from the same donor at stage 58 (beginning of metamorphosis period) with accelerated kinetics suggesting immunological memory similar to second set adult grafts. Therefore, *X. laevis* larvae are immunocompetent and can indeed be sensitized against the grafts. Also young adults (2 months post metamorphosis, postmetamorphic animals) similarly to larvae were able to completely and acutely reject sibling allografts in 100% of the cases with mean rejection time of 20 days (Table 2).

In contrast, during metamorphosis (which actually includes the time spanning 15 days before (st. 58) and about a month after metamorphosis, perimetamorphic animals) up to 50% of the sibling grafts were actually tolerized (i.e., not rejected), and those that were rejected followed a very slow rejection kinetics (20 - 80 days for complete rejection) in comparison to grafts on young larvae or adults (10 - 20 days). This suggests that metamorphosis is a special developmental time during which immune tolerance can be induced. This could be due to the fact that the immune system is undergoing complete remodeling and there is reduced number of lymphocytes. For example, more than 50% thymocytes die during metamorphosis (Du Pasquier and Weiss 1973). However, several pieces of evidence do not support the hypothesis that tolerance induction at metamorphosis is due to an insufficient number of lymphocytes. For instance, regardless whether the grafts are rejected or tolerized they are infiltrated by lymphocytes (Horton 1969; Bernardini, Chardonnens et al. 1970), while autografts are not. This implies that the grafts are recognized as non-self although they are not rejected. Also if tolerance is induced due to the lack of lymphocytes one would assume that once metamorphosis is completed and the number of lymphocytes is recovered, these grafts would be rejected. However, that is not the case since these grafts survive for more than two years. Interestingly, this tolerance can be broken by a third party graft (Bernardini, Chardonnens et al. 1970), which suggests that some H-Ags may be shared between the two different grafts. Therefore, once a T cell response is initiated against the third party graft that response can also be cross-reactive to the tolerized graft. Thus, it
appears that during metamorphosis *X. laevis* larvae are capable of inducing active allotolerance against certain H loci shared by the different outbred *X. laevis* used (Chardonnens and Du Pasquier 1973; Chardonnens 1975).

On the other hand, allograft tolerance capacity was found to occur also at premetamorphic stages (Table 2) when more genetically defined and homogeneous animals were used (DiMarzo 1980; DiMarzo and Cohen 1982a; DiMarzo and Cohen 1982b). Remarkably, both pre and perimetamorphic larvae of inbred strains were able to induce tolerance against allografts that were either minor H locus or even MHC-disparate, whereas all postmetamorphic froglets rejected 100% of the grafts (Table 2). In addition, differences in rejection depending on the developmental stage were noted; in general younger larvae (st. 47/48) had a higher propensity for becoming tolerant while older larvae (st. 57/58) had the ability to reject more grafts. In either case, the grafts were rejected with delayed kinetics in comparison to postmetamorphic animals. Despite the discrepancy on the ontogeny of allotolerance in *X. laevis*, it is clear that perimetamorphic larvae are indeed able to become tolerant to allografts in all cases. The inconsistency may be in part due to individual variation of thyroid hormone levels and the impact it has on initiation of metamorphosis. It is also possible that different tolerance mechanisms or regulatory cells are involved in premetamorphic and metamorphic stages.

### 4.2 Immune response and allotolerance in *X. laevis* larvae to MHC-disparate grafts

Although early experiments have indicated that during metamorphosis allotolerance is induced to minor H-Ags (Chardonnens and Du Pasquier 1973; Chardonnens 1975; DiMarzo 1980; DiMarzo and Cohen 1982a; DiMarzo and Cohen 1982b), other work has revealed that the immunogenetics of tolerance are complex. Family studies using field-collected outbred adult *X. laevis* showed that when grafted during metamorphosis skin that differed by one-MHC haplotype had prolonged survival (more than 75 days) and the majority of those grafts were never rejected suggesting long lasting tolerance (Barlow, DiMarzo et al. 1981). In contrast, all post metamorphic frogs rejected their grafts. Notably, grafts that were rejected by animals grafted during metamorphosis took at least twice as long for complete rejection. When two-MHC haplotype-disparate grafts were transplanted during metamorphosis the majority of grafts were rejected. However, even in this case, grafts did enjoy prolonged survival (more than 42 days for complete rejection in comparison to 18 days in postmetamorphic hosts, Table 2). In this study even though similar results were obtained using six different families, there was also variability from one family to another which suggests that genetics play a large role in the induction of tolerance (Barlow, DiMarzo et al. 1981).

In order to bypass undefined genetic variation, Barlow et al. (1981) used MHC defined inbred strains of *X. laevis*. In two different MHC combination when the donor and the recipient differed by one-MHC haplotype there was 93% graft survival in perimetamorphic hosts, while 100% grafts were rejected by postmetamorphic recipients (Table 2). When the graft differed by two-MHC haplotypes only 30% of grafts on perimetamorphic hosts survived more than 50 days. These data clearly show that during metamorphosis allotolerance can easily be induced to minor H-Ags as well as one-MHC haplotype-disparate grafts but rarely to grafts differing by two MHC haplotypes.

In addition to the genetic background of the grafts, as mentioned before, graft size plays an important role which can tip the balance between rejection and tolerance (Bernardini, Chardonnens et al. 1970; Chardonnens and Du Pasquier 1973; Barlow and Cohen 1983).
general smaller grafts (1-2 mm²) are more readily rejected (can reach up to 90% rejection) in perimetamorphic animals when there is a one MHC haplotype difference between donor and host. On the other hand, larger grafts (4-9 mm²) almost always induce tolerance. The same trend holds true for grafts differing by two MHC haplotypes except the capacity for rejection is greater since tolerance is not easily induced in this case.

4.3 Mechanisms of allotolerance

Cell transfer approaches were developed to investigate the cellular mechanism responsible for tolerance induction and maintenance of skin Ags in *X. laevis*. In a set of experiments, isogenic frogs carrying minor H-Ag-disparate grafts were injected with thymocytes and splenocytes from isogenic metamorphosing larvae. This cell transfer induced significantly delayed skin graft rejection in comparison to control animals that received either adult cells or were not adoptively transferred with any cells (Du Pasquier and Bernard 1980). Importantly, multiple injections of metamorphosing immune cells were necessary to achieve suppression of graft rejection since a single injection did not result in the specific delay. Additionally, this effect was not permanent; once the injections stopped the grafts were rejected. Similarly, splenocytes from larvaly tolerant frogs that were adoptively transferred into normal frogs drastically suppressed the rejection of semi-allogeneic grafts (Nakamura, Maeno et al. 1987). This indicates that the thymus and spleen of metamorphosing animals contain cells, presumably lymphocytes that have tolerogenic or suppressive activity against skin minor H-Ags. The need for repeated transfers of these cells to delay graft rejection may be due to their low frequency and/or short life span in the spleen and thymus of the metamorphic donor or as a result of the cell transfer. Additionally, the fact that the effect does not last suggests that tolerance induction to skin grafts during metamorphosis is regulatory (requiring the presence of regulatory cells) rather than deletional (absence or deletion of potentially reactive T cells). This possibility is further supported by experiments showing that maintenance of tolerance to minor H-Ag-disparate skin grafts can be broken by cyclophosphamide treatment (Horton, Horton et al. 1989).

In a complementary set of experiments, adoptive cell transfer was used to determine if tolerance to skin minor H-Ags can be broken (Du Pasquier and Bernard 1980). Splenocytes from isogenic adults primed by minor H-Ag-disparate skin grafts were adoptively transferred into isogenic metamorphic recipients with tolerized skin graft genetically identical to the one used to prime the adults. This adoptive transfer of primed anti-minor H-Ag lymphocytes was not able to break tolerance in animals that tolerated the grafts for at least six months. However, if a second graft, identical to the tolerized graft, was placed at the time of the adoptive transfer, an acute rejection of this graft was initiated but stopped within 10 days. The result was a graft that was half rejected and half healthy. This suggests that even though the animal is tolerant to the allograft, the primed transferred cells are able to cause graft destruction although the number and/or survival of these reactive cells is not sufficient to complete the rejection. Presumably, if another transfer was done the graft may be fully rejected. Another possibility is that a subset of regulatory cells home to the skin at the time of transplantation to induce and maintain tolerance or suppression, and that the presence of adoptively transferred anti-minor H-Ag lymphocytes prevent or delay the migration of these regulatory cell in the transplanted skin. Taken together, these data strongly suggest that tolerance in larvae does not depend upon deletion of alloreactive cells but rather is maintained by suppressor or regulatory cells that can be adoptively transferred.
The preponderant role of T cells in larvally-induced allotolerance has been established by thymectomy. When larvae were thymectomized during early larval life before the migration of T cell precursors into the thymic anlage, graft rejection of premetamorphic larvae as well as postmetamorphic froglets was severely impaired (Table 2) demonstrating that without the thymus alloreactive cells can not develop (Horton and Manning 1972; Barlow and Cohen 1983; Kaye and Tompkins 1983; Nagata and Cohen 1983). Furthermore, the impaired rejection capacity of premetamorphic larvae that were thymectomized at early developmental stage can be rescued by implantation of an intact larval or adult thymus (Arnall and Horton 1986). When these T cell deficient thymectomized larvae are implanted with an isogenic thymus their capacity to reject both minor H-Ags and MHC-disparate skin grafts was fully restored. However, when thymectomized larvae were reconstituted with a thymus that was either MHC-disparate or differed even by minor H-Ags from the host, rejection was restored only to grafts that were MHC incompatible both to the host and the thymus donor. Interestingly, these reconstituted animals had an impaired ability to reject minor H-Ag-disparate grafts. Notably, when MHC incompatible thymi were transplanted, skin grafts from the same donor were tolerated even though they were able to elicit proliferative responses against the thymus donor cells in an in vitro MLR (Arnall and Horton 1986). This split tolerance was further substantiated in vivo by injecting irradiated splenocytes from the thymus donor into reconstituted animals (Arnall and Horton 1987). Again there was an in vivo response against the transferred splenocytes, although the grafts were still not rejected. In addition, if live MHC-disparate splenocytes from the thymus donor were injected, animals developed graft versus host disease and died. Based on these results it has been concluded that the cytotoxic rather than the proliferative alloresponse is suppressed in thymus reconstituted thymectomized animals. In contrast to early thymectomy, late larval thymectomy did not have an effect on rejection (Table 2). Interestingly, however, thymectomy performed during mid-larval stages significantly impaired the ability of animals to become tolerant to allografts (Table 2) (Barlow and Cohen 1983). Moreover, this impairment was dependent on the number of different MHC haplotypes as well the size of the grafts. In conclusion, it is clear that X. laevis larvae can become tolerant to allografts although the developmental stage at which this tolerance can be established, the types and amount of skin Ags that can be tolerized, and the cellular mechanisms involved still remain unclear. During metamorphosis the larval immune system is drastically remodeled and larvae develop tolerance against newly expressed adult self-antigens; therefore, allotolerance may be a consequence of this period. This does not mean that there are not enough effector cells or CTLs to reject the grafts since it was clearly demonstrated that MLR in these animals was positive meaning that T cells can proliferate in the presence of alloantigens. A most likely scenario is that during metamorphosis there is a general suppressive state where putative T regulatory cells suppress immune responses against adult tissues in order to avoid autoimmunity. The thyroid hormone signaling which initiates metamorphosis may be one plausible factor of individual variability for premetamorphic larvae to become tolerant to allografts. As explained earlier, thyroid hormone acts locally and it is possible that in some cases animals start producing larger quantities of this hormone in certain tissues such as the skin, which would mean that even if the animal is not showing general changes associated with metamorphosis certain organs or tissues may be. It is possible that particular organs or tissues will be associated with a more suppressive environment suited for induction of
tolerance. Although, the tolerance ability of premetamorphic stages may result from the establishment of this regulatory process that would peak during the metamorphic climax, it is also possible that it results from a different mechanism such as insufficient or immature T cell response. This can especially be the case at earlier larval stages (eg. st. 53) when peripheral T cells are still few (~10,000 to 100,000).

<table>
<thead>
<tr>
<th>Developmental Stage</th>
<th>Premetamorphic</th>
<th>Perimetamorphic</th>
<th>Postmetamorphic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minor-H-Ag disparate grafts</td>
<td>Tolerance induction +*</td>
<td>Tolerance induction +++</td>
<td>Rejection 100%</td>
</tr>
<tr>
<td>1 MHC haplotype disparate grafts</td>
<td>Tolerance induction +*</td>
<td>Tolerance induction +++</td>
<td>Rejection 100%</td>
</tr>
<tr>
<td>2 MHC haplotypes disparate grafts</td>
<td>Rejection</td>
<td>Rejection MST 42</td>
<td>Rejection MST 18</td>
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<tr>
<td>Early larval life thymectomy</td>
<td>Impaired graft rejection</td>
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<td>Impaired graft rejection</td>
</tr>
<tr>
<td>Mid larval life thymectomy</td>
<td>No effect</td>
<td>Impaired tolerance</td>
<td>No effect</td>
</tr>
<tr>
<td>Late larval life thymectomy</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
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Table 2. Summary of tolerance induction in X. laevis larvae and the effects of thymectomy.

*Discrepancy between different studies. Chardonnens and Du Pasquier (1973) reported that premetamorphic larvae were capable of rejecting grafts similarly to postmetamorphic froglets while several reports from the Cohen lab reported that premetamorphic larvae (as early as stage 47) had the capacity to induce tolerance. +, low incidence of tolerance induction +++, high incidence of tolerance induction MST: Mean Survival Time (in days)

4.4 New tools to study tolerance

During metamorphosis, as described before, X. laevis larvae have to become tolerant to newly emerging adult self-antigens, which can be recognized as foreign by the larval immune system, and lead to autoimmunity. It is highly plausible that the same mechanisms that are involved in allotolerance are also involved in tolerance to self; therefore, X. laevis provides a unique model to study autoimmunity. This is illustrated by the observation that certain animals that are thymectomized at stage 56 and then transplanted with MHC-disparate graft have a high incidence of death due to “red disease” (Barlow and Cohen 1983). This is a disease that is autoimmune in nature and is characterized by cutaneous
hemorrhaging around the eyes and legs and by internal hemorrhaging in the liver and kidneys. There is still much unknown regarding the mechanism(s) associated with suppression during allotolerance. For instance, we still don’t know which effector cells are involved, even though we speculate that they might be T regulatory cells. The mode and site of action of these regulatory cells is also not defined. Using some newly generated tools and techniques we can start to answer some of these questions. Initially, it will be of great interest to immunize perimetamorphic larvae with hsps carrying either MHC or minor H-Ags and then look for immunological memory toward skin grafts (same genetic background as the Ags carried by the hsp) transplanted post metamorphosis. These experiments will show if the Ags alone are capable of generating tolerance by possibly negatively selecting reactive thymocytes in the thymus or by generating specific T regulatory cells toward those particular Ags.

To further investigate the effector cells involved in these responses, we will be able to use transgenic animals. Transgenesis in X. laevis has been widely used over the last several years and the most common technique used is REMI (Restriction Enzyme Mediated Integration) which requires integration of the transgene into the sperm nuclei which are than transplanted into unfertilized eggs (Kroll and Amaya 1996). However, for generating transgenics with our isogenic clones this technique is not useful since they are maintained by gynogenesis. Therefore, our laboratory adapted a new transgenic approach using the I-SceI Meganuclease (Ogino, McConnell et al. 2006; Pan, Chen et al. 2006). This method requires a plasmid that carries the transgene of interested flanked by I-SceI recognition sites which is digested by the meganuclease and the entire digest is injected into activated eggs. This causes stable integration of the transgene into one to two different sites in the genome. An added advantage of working with the clones is that all of the progeny of a given founder will be transgenic which will give us enough larvae for experimentation.

As mentioned in the introduction the genome of the X. laevis sister species, X. tropicalis has been fully sequenced and annotated. This has provided new possibilities to identify immune genes using, for example, gene synteny (Robert and Ohta 2009). Furthermore, the full sequencing of the X. laevis genome using the homozygous inbred strain J from our resource center is now ongoing and the first assembly is already available (R. Harland, personal communication). With these new available resources, it will become possible to identify and isolate regulatory regions of immunologically relevant genes and produce transgenic reporter animals expressing fluorescent reporter genes (such as GFP) under the control of these regulatory regions as in mouse and zebra fish (Smith, Ataliotis et al. 2005; Doherty, Johnson Hamlet et al. 2007; Hall, Flores et al. 2009). X. laevis transgenic lines expressing, for example, GFP under the transcriptional control of the CD4 (CD4 T cells) or the Foxp3 (T regulatory cells) promoter regions (i.e., homologs of these genes have already been identified in the X. tropicalis genome), would permit to localize and follow the fate of these cells in transplanted skin tissues during rejection and tolerance induction. Since tadpoles are transparent this will allow us to easily visualize the trafficking pattern of these cells and see if those are the effector cells that are found infiltrating tolerated grafts. Also we can sort these cells based on their GFP expression and then adoptively transfer them into postmetamorphic frogs to check if indeed these cells are involved in the induction of tolerance. Additionally, by using cloned animals we will be able to pool enough larval cells
to perform the adoptive transfers as well as to use multiple transfers to see if the number of transferred cells will have an effect on allotolerance.

Another area of investigation where *X. laevis* may reveal useful involves the possible role of certain class Ib molecules in tolerance induction. In mice and humans certain NKT cells that are educated onto class Ib molecules seem to be involved in autoimmunity. The partial characterization of skin γδ T cells as well as dendritic cells has recently been reported in adult *X. laevis* (Mescher, Wolf et al. 2007). It would be interesting to determine the similarity and potential difference in the larval skin, since old data suggest that in contrast to adult, larval skin does not have Langerhan cells (Du Pasquier and Flajnik 1990). Furthermore, since *X. laevis* tadpoles do not have consistent class Ia expression until metamorphosis their T cells may be educated on class Ib molecules that are expressed in the thymus during early development. We can test if certain class Ib molecules are involved in tolerance induction by down regulating these genes in vivo by transgenesis. We have already established a protocol to silence *X. laevis* genes, including class Ibs, using RNA interference (Goyos, Guselnikov et al. 2007; Nedelkova and Robert unpublished). We can also generate specific shRNAs targeting genes critical for T regulatory cell function to determine if impairment of this cell type will lead to loss of tolerance and induction of autoimmunity.

5. Conclusion
The studies reviewed here highlight the versatility and attractiveness of *X. laevis* to study skin graft rejection as well as immune tolerance. Due to the dual nature of its immune system, larval and adult, *X. laevis* can be used as a useful model for investigating T cell regulation as well as long term immunological memory. Furthermore, this system already demonstrated and will further explore the conserved ability of heat shock proteins to elicit Ag specific CD8 T cell responses. Finally, *X. laevis* provides a powerful model to study self-tolerance by dissecting the mechanisms involved in induction of allotolerance. The advent of new genetic and genomic tools and technologies will allow better insight into the complex regulation and development of allotolerance which is of general relevance for generating self-tolerance, as well as autoimmunity.

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Comparative Study of Skin Graft Tolerance and Rejection in the Frog *Xenopus Laevis*


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Comparative Study of Skin Graft Tolerance and Rejection in the Frog *Xenopus Laevis*


The procedure of skin grafting has been performed since 3000BC and with the aid of modern technology has evolved through the years. While the development of new techniques and devices has significantly improved the functional as well as the aesthetic results from skin grafting, the fundamentals of skin grafting have remained the same, a healthy vascular granulating wound bed free of infection. Adherence to the recipient bed is the most important factor in skin graft survival and research continues introducing new techniques that promote this process. Biological and synthetic skin substitutes have also provided better treatment options as well as HLA tissue typing and the use of growth factors. Even today, skin grafts remain the most common and least invasive procedure for the closure of soft tissue defects but the quest for perfection continues.

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