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Self/nonself discrimination at the basis of chordate evolution: limits on molecular conservation

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All organisms rely on their capacity of self/nonself discrimination to rapidly detect approaching allogeneic cells as well as invading pathogenic microbes as foreign and to eliminate them. Failure to recognize nonself causes self-mating, germline parasitism and disease. Recent findings indicate that, in urochordates — the closest living relatives of vertebrates — different species use completely different molecules for allorecognition. Thanks to their phylogenetic position, these organisms might help us to understand the evolutionary origin of the vertebrate immune system.

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Introduction

The capacity to discriminate between self and nonself is a fundamental requirement of life [1••]. The innate immune system employs pattern recognition receptors (PRRs) to recognize conserved pathogen-associated molecular patterns (PAMPs) that distinguish foreign organisms — viruses, bacteria, fungi and parasites — from cells of their hosts [2–6]. This system is evolutionary conserved and functions by eliminating ‘by default’ all cells that do not possess the correct ‘self markers’. Unlike innate immunity, adaptive immunity (which only evolved in vertebrates) is based on cell-surface receptors (generated by somatic gene rearrangements) that recognize an infinite variety of antigens. The adaptive immune system is based on the recognition and elimination of all possible ‘nonself’ rather than on detection of typical PAMPs or self markers, and typically involves the major histocompatibility complex (MHC), T-cell receptors (TCRs) and antibodies. In jawed vertebrates and especially in mammals, for which

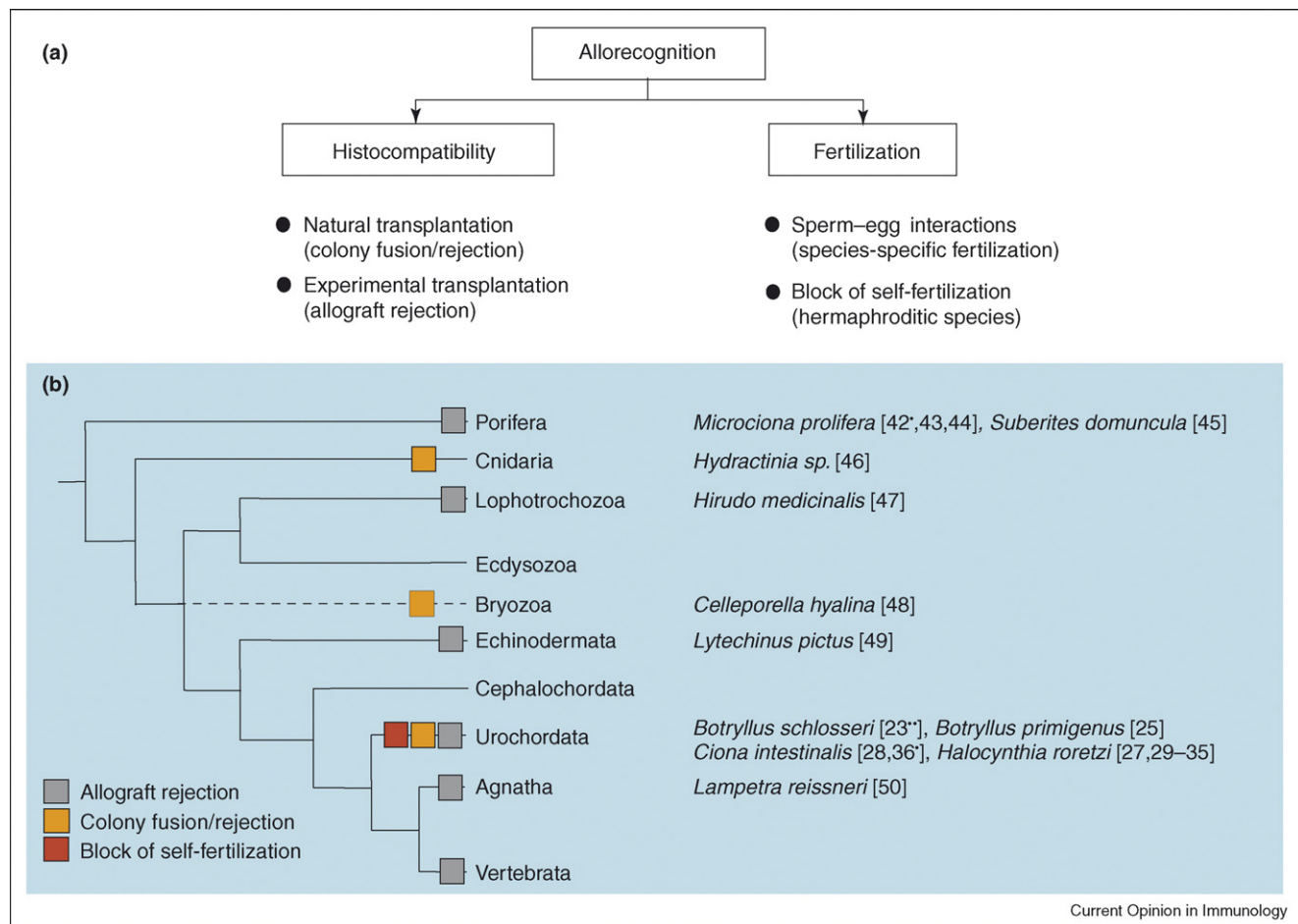
most of the data are available, both the adaptive and innate immune counterparts are highly integrated. Crucial for this integration appear to be the natural killer cells, which not only utilize the ‘missing self’ concept typical for innate immunity but also screen the surfaces of body cells for the presence of receptors of the adaptive immune system (e.g. HLA-E), which are in this case utilized as self markers.

Self/nonself discrimination is crucial for several diverse biological events. In addition to rapidly detecting invading pathogenic microbes, self/nonself discrimination is essential for the sperm–egg interaction to ensure species-specificity during fertilization (Figure 1a). In hermaphroditic animals (e.g. flat worms, annelids and solitary ascidians), which produce sperm and eggs simultaneously, additional recognition mechanisms must exist to prevent self-fertilization. Moreover, colonial animals (e.g. bryozoans and compound ascidians) have to rely on their capacity to distinguish between self and nonself to prevent uncontrolled fusion of genetically different individuals. In mammals, this type of ‘natural transplantation’ never occurs. However, if tissue from one individual is transplanted to another genetically unrelated individual it will be rejected. This ability to distinguish conspecific but genetically different tissue as nonself is generally referred to as allorecognition (Figure 1a). Although in vertebrates it is known that allorecognition (here also frequently termed histocompatibility) is a side effect of the ability of the adaptive immune system to recognize ‘nonself’ with the help of TCRs and MHC, the mechanisms of allorecognition used by animals at the origin and early evolution of vertebrates are poorly understood. Comprehensive expressed sequence tag (EST) datasets and genome projects in several taxonomic groups [7–10] indicate that MHC and TCR are not present outside jawed vertebrates. Thus, the ability for allorecognition in these animals must rely on molecular mechanisms different from those of jawed vertebrates.

In this review, we will discuss novel insights into the molecules mediating allogeneic recognition in urochordates — the closest living relatives of vertebrates — according to recent phylogenetic analyses [11,12]. Most importantly, it has become obvious that, in even closely related species of urochordates, different receptors play distinct roles in distinguishing between self and nonself. None of the molecules used for allorecognition in urochordates appear to be involved in self/nonself discrimination in other animals.

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Figure 1



The ability to distinguish conspecific but genetically different tissue as nonself (alloreognition) is crucial for several biological processes.

(a) Allogeneic recognition (alloreognition) can be divided into tissue histocompatibility and recognition events during gamete interactions that precede fertilization. Various modes of alloreognition are unequally distributed within the animal kingdom. Although correct recognition during fertilization is crucial for all animals, natural transplantation occurs only in colonial animals. Only in vertebrates is the molecular basis of histocompatibility known and connected with the function of the immune system. To what extent alloreognition is based on components of the immune system in invertebrates remains unclear. **(b)** Allograft rejection, colony fusion and block of self-fertilization in different taxonomic groups. The representatives of each animal group in which alloreognition reactions were studied are listed, with the references in square brackets [23*,25,27–35,36*,42*,43–50].

Recognition of nonself in the colonial urochordate *Botryllus schlosseri*

Colony fusion or rejection in *Botryllus schlosseri* was first detected by Bancroft more than 100 years ago [13]. According to his report, when two pieces of a single colony came into contact with each other, they easily fused to form a single colony. Two pieces of different origin, however, did not fuse after grafting, regardless of conditions. Later on, Oka and Watanabe [14,15] proved the genetic basis of this phenomenon in the Japanese species *Botryllus primigenus*. The ability to fuse or to reject was shown to be inherited according to simple Mendelian rules, indicating that a single genomic locus (termed fusibility/histocompatibility, FuHc) is responsible for alloreognition in *Botryllus* [16–18].

Almost 35 years ago, Burnet [19] proposed that the alloreognition systems responsible for tissue fusion or rejection in colonial invertebrates (cnidarians, bryozoans and urochordates) might be the antecedents of mammalian histocompatibility. Since then, colonial urochordates of the genus *Botryllus* have become one of the main model systems to study allogeneic recognition in invertebrates. At the beginning, taking into consideration the phylogenetic proximity of vertebrates and urochordates, it was anticipated that alloreognition in *Botryllus* is governed by molecules homologous to mammalian HLA. In fact, the *Botryllus* FuHc locus itself was considered a simplified version of the mammalian MHC [16,18]. Since then, there have been many attempts to identify MHC-like molecules in *Botryllus* by homology

hunting, but none have been successful to date (reviewed in [20]).

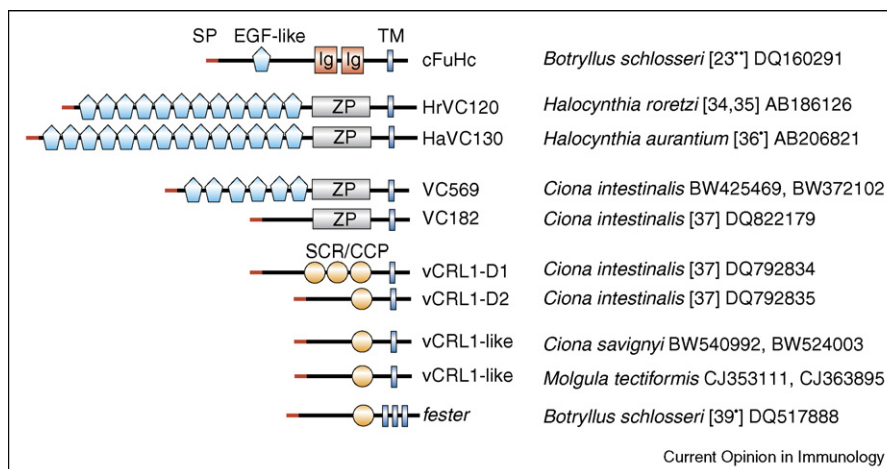
Most recently, the *Botryllus* FuHc locus was delineated by an amplified fragment length polymorphism-based genetic approach, resulting in a region of about 600 Kbp being physically isolated and fully sequenced [21,22]. This technically challenging, but beautiful and unbiased, approach revealed why all attempts of homology hunting were in vane: the FuHc locus of *Botryllus* does not contain any gene homologous to HLA and there is no synteny to the MHC region of any vertebrate. Unexpectedly, it turned out that *B. schlosseri* uses its own 'invention' for control of colony fusion — a transmembrane protein that contains one epidermal growth factor (EGF)-like domain and two immunoglobulin-like domains (Figure 2; [23**]). The receptor termed cFuHc varies in sequence between individuals. Every two individuals that share at least one allele will fuse, whereas individuals who do not share any cFuHc alleles will reject. In natural populations, hundreds of different cFuHc alleles are present. Therefore, the probability of fusion of genetically unrelated colonies is negligibly low, and maintenance of individuality is guaranteed. cFuHc is not homologous to any of molecules of the vertebrate MHC-based histocompatibility system [23**,24]. Most interestingly, the genomes of the solitary urochordates *Ciona intestinalis* and *Ciona savignyi* do not contain homologs of the *Botryllus* cFuHc gene. Moreover, the whole *Botryllus* FuHc locus does not have a syntenic region in the *Ciona* genome or in the genomes of vertebrates [22,23**].

In addition to controlling colony fusion, urochordates (as simultaneous hermaphrodites) require efficient mechanisms to prevent self-mating. Interestingly, in *B. primigenus* the loci responsible for rejection of allografts and for prevention of self-mating appear to be genetically linked (reviewed in [25]). In *B. schlosseri*, however, the role of the FuHc locus in fertilization is controversially discussed: whereas Scofield *et al.* [16] reported the existence of self-sterility in *B. schlosseri*, other researches [26] found no evidence for a role of the FuHc locus on fertilization. Because these discrepancies might also be caused by differences in the animal lines used for the experiments, the molecular basis responsible for block of self-fertilization in *Botryllus* remains to be elucidated.

Recognition of self to prevent self-fertilization in the solitary urochordates *Halocynthia roretzi* and *Halocynthia aurantium*

Solitary urochordates are well established models to study systems of self/nonself recognition that prevent self-mating [27,28]. In *Halocynthia roretzi*, for example, under normal conditions sperm can never fertilize eggs from the same individual. The fertilization process in this species was extensively studied at the morphological and histological level [27] and several molecules involved (e.g. sperm lysins and proteosomes) were characterized in detail [29–33]. Interestingly, *H. roretzi* eggs are not self-sterile by default. The self-sterility is acquired during a maturation process before gamete spawning. Major players in the onset of self-sterility are follicle cells, which surround the mature oocyte and produce and deposit 'self-sterility' factor(s) onto the vitelline coat of the

Figure 2



Variable genes in urochordates that were proposed to be involved in allorecognition. Although the cFuHc gene from *Botryllus* [22,23**] and ZP-containing genes in *Halocynthia* [34,35,36*] do not resemble any molecules in the vertebrate innate immune system, vCRL1-like genes from *Ciona* [37] and *Molgula* and the *fester* gene from *Botryllus* [39] are structurally similar to vertebrate complement receptors CD46 and CD55. Thus, at the basis of chordate evolution allogeneic reactions might be based on molecules that originate from defence mechanisms of the innate immune system. GeneBank accession numbers for putative allorecognition proteins are given, and references are provided in square brackets. Abbreviations: EGF, epidermal growth factor-like domain; SCR, short consensus repeat; SP, signal peptide; TM, transmembrane domain; ZP, zona pellucida domain.

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maturing oocytes. Oocytes deprived of follicle cells (by treatment with acidic sea water) become self-fertile [27].

One of the advantages of *Halocynthia* is that a large number of eggs can be harvested from a single individual. This fact facilitated the recent isolation of a putative self-sterility receptor by an unbiased biochemical approach [34,35]. When comparing the components of the vitelline coats from immature oocytes with those from mature oocytes of *H. roretzi*, Sawada *et al.* [35] discovered that the amount of a 70 kDa vitelline-coat protein markedly increased during oocyte maturation. The protein is deposited onto the vitelline coat by follicle cells and is quickly degraded upon treatment of *H. roretzi* eggs with acidic sea water. This putative self-sterility protein is a transmembrane receptor termed HrVC120 that is expressed on oocytes, which has 12 EGF-like domains and one zona pellucida (ZP) domain (Figure 2; [35]). EGF-like repeats in HrVC120 show genetic polymorphism between *Halocynthia* individuals. In the closely related species — *Halocynthia aurantium* — the homologous receptor HaVC130 (see Figure 2) was identified and variable sites were shown to be under diversifying selection [36]. The number of EGF-like domains in HrVC120 and HaVC130 differ but, on the basis of amino acid sequence conservation, these proteins are clearly homologous.

Thus, as indicated in Figure 2, allrecognition molecules appear to be highly conserved within the genus *Halocynthia*. Interestingly, the genome of the urochordate *C. intestinalis* does not contain any homolog of HrVC120. *Ciona* contains, however, several unrelated transmembrane proteins that have several EGF-like domains followed by the ZP domain. We have identified recently at least five *Ciona* proteins that have domain organization such as this (see Figure 2 [37]; U Kürn and K Khalturin, unpublished). According to our data, these proteins are expressed in developing oocytes and no transcripts can be detected in mature oocytes or follicle cells (U Kürn and K Khalturin, unpublished). The amino acid sequences of these proteins are variable from individual to individual, similar to HrVC120. Although these observations are consistent with a role for proteins that have multiple EGF-like domains and a ZP domain in urochordate fertilization, they are, however, not definitive in revealing the function of these variable receptors.

Recognition of self to prevent self-mating in the solitary urochordate *Ciona intestinalis*

The solitary urochordate *Ciona* is able to distinguish between self and nonself to prevent self-fertilization [28] and to reject allografts [38]. The genomes of both *C. intestinalis* and *C. savignyi*, however, do not contain homologues of either the *Botryllus* cFuHc protein or the putative self-sterility receptor of *Halocynthia* [37]. How does *Ciona* distinguish between self and nonself? It is

obvious that these animals must have invented their own tool-kit for self/nonself discrimination.

Molecular markers of 'self' are expected to be highly variable between individuals within one species. To search for variable proteins taking part in *Ciona* self-sterility, we compared the transcriptomes from female gonads of three *C. intestinalis* individuals by suppression subtractive hybridisation (SSH) [37]. The goal was to identify transcripts that vary between individuals and therefore code for variable proteins that could be used as 'self' markers. The screening led to the identification of several variable proteins [37]. The most remarkable among them is a transmembrane protein that has three short consensus repeats (also known as complement-controlling protein) domains termed variable complement receptor-like protein 1 (vCRL1) (Figure 2). vCRL1 not only is strikingly variable between *Ciona* individuals, showing only 75–85% identity on amino acid level between randomly selected individuals, but also is expressed in follicle cells and hemocytes. The extraordinary high variability and the expression in follicle cells, which are responsible for the onset of self-sterility, are consistent with a crucial role of vCRL1 in self/nonself recognition during fertilization [37].

Although the *Ciona* vCRL1 protein is not homologous to HrVC120 from *Halocynthia*, a clearly homologous vCRL1 protein was identified in *C. savignyi* EST and genome projects (BW540992, BW534157, BW563675, BW524003, BW573678 and BW513196) as well as in an EST project of *Molgula rectiformis* (CJ353111 and CJ363895) (see Figure 2). We also note that the FuHc locus of *B. schlosseri* contains several genes that encode transmembrane proteins that have short consensus repeats domains (scaffolds AC138583 and AC140856). The sequence similarity between them and *Ciona* vCRL1 does not allow any conclusions about a common evolutionary history, but one of these genes, termed *fester* (DQ517888; Figure 2), was proposed to be a histocompatibility receptor in addition to cFuHc [39]. The detailed function of these proteins remains to be elucidated in the future.

The identification of a variable complement receptor-like protein as a putative self-sterility receptor in *Ciona* has an interesting evolutionary implication. In the immune systems of vertebrates, complement receptors are known to protect cells against inappropriate activation of their own complement system. Therefore they act as 'inhibitors' of the complement and mark cells as self. The complement system is one of the most conserved parts of the immune system throughout the animal kingdom [40,41]. Thus, it is intriguing to hypothesize that, at the base of chordate evolution, the self-sterility machinery evolved from a defence pathway by co-opting a subset of complement molecules to accomplish self-recognition during fertilization.

Conclusions

It seems that urochordates possess a relatively complex self/nonself discrimination system that is without analogy to the known self discrimination systems in other animals and, therefore, has evolved independently. There is emerging evidence that three representatives of the phylum Urochordata (*C. intestinalis*, *B. schlosseri* and *H. roretzi*) recruit three completely different molecules to distinguish self from nonself. An apparently similar situation can be found in the mechanisms of self-incompatibility in flowering plants. In this situation, for example, receptors and ligands of the S-locus in Brassicaceae are different from the self-incompatibility molecules in Papaveraceae, and this variety is increasing as additional plant taxa are studied (reviewed in [42*]).

We predict that, with the recent advances of genomics, even in non-model organisms, more unique and specialized self discrimination systems will be discovered. The challenge of the next few years will be to understand how the astonishingly large interindividual variability of ligands and receptors allows organisms to maintain stable interactions between a particular receptor and ligand. Comparative analyses that address this will shed new light on the evolutionary history of self/nonself discrimination systems.

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