Chimerism a natural ability to tolerate kin, evolutionary traits connecting mammalian and protochordates

A Voskoboynik

Institute of Stem Cell Biology and Regenerative Medicine, Department of Pathology, Stanford University School of Medicine, Stanford, USA and Department of Developmental Biology, Stanford University Hopkins Marine Station, Pacific Grove, USA

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Abstract

In the middle of the 20th century, Owen (1945, 1954) and Billingham et al. (1953) immunological studies suggested that fetal exposure to foreign antigens during pregnancy induce immunologic tolerance in the fetus. Recently, Mold et al. found that a substantial number of maternal cells crosses the placenta to reside in fetal lymph nodes and induces the development of regulatory T cells (Tregs) that suppress fetal anti-maternal immunity. These Tregs cells persist till, at least, early adulthood. This result demonstrates how chimerism induces fetal tolerance to maternal antigens during mammalian pregnancy. Natural chimerism is the coexistence of two or more genomic lineages within the same individual. It is a common phenomenon which can be detected in a wide variety of multi-cellular organisms. In mammals, natural chimerism can be established during pregnancy between the mother and the fetus or between fetuses in a multiple embryos pregnancy. Restriction of natural chimerism mainly to kin is also observed in colonial marine protochordates. In protochordates, like Botryllus schlosseri, natural chimerism can be established through fusion of vasculature, between a parent colony and its progeny or between siblings (adult distinct colonies). The ability to tolerate a partial allogeneic individual and to create a chimeric entity between these colonies is determined by a single, highly polymorphic, fusion/histocompatibility locus (Fu/HC). Colonies that share at least one allele in their Fu/HC locus would fuse upon contact. A pair that does not share any Fu/HC allele would not. In the chimera, cells transmigrate between partners and in some cases, replace the germline and/or the somatic tissues of the host. This genotype replacement is mediated by stem cells (termed somatic/germ cell parasitism). Botryllus colonies propagate asexually through budding, therefore somatic stem cell parasitism in host colonies can induce the development of a partial allogeneic entity (buds) within the host colony. In this way, chimerism in protochordates serves as a state that enables the development of a "virtual embryo" within the host colony. In light of Mold et al., study, which demonstrates a role to chimerism in tolerance induction during pregnancy, studying the immunological mediators for natural acceptance of partial allogeneic allograft in protochordates may reveal the evolutionary precursors to the tolerance state during mammalian pregnancy.

Key words: chimerism; immunologic tolerance; stem cells; tunicate; mammalian pregnancy; Fu/HC; uterine NK

Chimerism in pregnancy

An increasing number of studies have detected natural chimerism in a wide variety of multi-cellular organisms, including vertebrates. This suggests that chimerism is a common phenomenon in the wild (e.g. Buss, 1982; van Dijk et al., 1996; Bianchi et al., 1996; Marleau et al., 2003; Pineda-Krch and Lehtila, 2004; Rinkevich 2004a, b; Kaplan and Land, 2005; Khosrotehrani et al., 2005; Loubiere et al., 2006; Bianchi, 2007; Mold et al., 2008). Studies demonstrated that chimerism can be experimentally initiated by engraftment of a single stem cell (e.g. Cao et al., 2004; Laird et al., 2005). Human chimerism which originated from twins was reported already in 1953. With the advent of blood typing, it was discovered that some people have blood cells...
of more than one type (Dunsford et al., 1953). Until 1996, twins chimerism seems to be very rare in humans, only 40 cases of twins chimerism were reported (Trippet, 1983). These cases were found during routine blood grouping, a procedure which detects a mixture of red blood cells only when the percentage of alloimmune cell in the blood is above 5% of all blood cells (van Dijk et al., 1996). van Dijk et al. (1996) applied a more sensitive method and found that in humans, 8% of non-identical twins and 21% of triplets are chimeric (van Dijk et al., 1996). Twin chimerism with high levels of blood cells from the other twin yields tolerance to donor antigens. All chimeras twins which were identified in a routine blood grouping (frequency >5% of chimeric cell population) had mutual tolerance to blood transfusion and skin grafts from their chimeric partner (Trippet, 1983). Chimeras with low frequencies of foreign cell population (like those identified by van Dijk et al. (1996), with chimeric cell population frequency of ~0.1%) were not tolerant to their twin foreign antigens and rejected blood grafts.

Fetal-maternal micro-chimerism is another natural chimerism that is developed during human pregnancy. Cell trafficking, during normal human pregnancy, between the mother and the fetus, leads to the establishment of a chimeric state in both. Fetal cells were identified in the maternal circulation (Herzenberg, 1979; Lo et al., 1989, 1996, 1998; Petit et al., 1997; Bianchi et al., 1997, 2002). Maternal cells were found in the umbilical cord and in fetal blood samples (Socie et al., 1994; Hall et al., 1995; Lo et al., 1996; Petit et al., 1995; 1997; Bauer et al., 2001). During human pregnancy, 20 %–50% of the erythroblasts in maternal blood are originated from the fetal (Sekizawa et al., 1996; von Eggeline et al., 1997; Oosterwijk et al., 1998; Wachtel et al., 1998; Troeger et al., 1999). These percentages indicate relatively high levels of chimerism between mother and fetus during pregnancy in the host mother. Persistence of alloimmune fetal progenitor cells were detected in chimeric mothers (humans) as long as 27 years after birth (termed as microchimerism, MC; Bianchi et al., 1996). Fetal mesenchymal stem cells were detected in maternal bone marrow and in mother’s ribs even 35 years after delivery (1 fetal cell in ~10^{5} maternal cells; O’Donoghue et al., 2004a, b). Long term persistence of maternal cells in progeny (maternal Mc) was described too (Maloney et al., 1999). Loubiere et al. (2006) found that high percent of healthy adult women harbor maternal and fetal Mc among their T and B lymphocytes, monocyte/macrophages and NK cells. 78 % (21/27) of healthy tested women carried fetal Mc (tested women’s children average age 12.6 years), 39 % carried maternal chimerism (12/31 average age = 39; Loubiere et al., 2006). Maternal / fetal MC is not limited to blood cells, it has been identified in many organs including normal kidney, liver and heart of women with or without pregnancy history (chimerism in different organs was detected in 23 out of 75 tested women, ages 29-93 at death; Koopmans et al., 2005). Since stem cells are the only cells in the tissues with a self-renewing capability, the detection of maternal and fetal MC up to four decades following delivery indicates that healthy humans harbor long-surviving populations of maternal/fetal hematopoietic stem cells. The above studies suggest that during pregnancy fetal/maternal stem cells enter maternal/fetal blood engraft, differentiate in host tissues and remain presence throughout its entire life.

Tolerance in pregnancy

Acceptance of a fetus, which expresses paternally inherited alloantigens, by the mother during pregnancy is a unique example of the way immune system reshapes a destructive alloimmune response to a state of tolerance (Guleria and Sayegh, 2007). Intolerance of the embryo, as an antigenically foreign body growing within the female, can represent a significant obstacle to reproduction. In humans, it is estimated that 70% of conceptions fail (Hill, 1992). Similar estimates of early fetal loss, ranging from 10% to 60% have been obtained for other mammalian species (Baker and Bellis, 1995). In human, approximately 45% of the couples experiencing primary recurrent spontaneous abortion have been linked to immunological factors (Clark, 1999). On the other hand, during successful mammalian pregnancy, the semiallogeneic fetus resides comfortably within the maternal uterus and is protected from response of maternal graft rejection. The maternal immune system is active during pregnancy, yet mothers tolerate and do not reject their genetically disparate fetuses (Hunt et al., 2005; Lightner et al., 2008). There is growing evidence that viviparous reproduction depends on a two-way interactions between fetal and maternal tissues that involve humoral, cellular, innate and adaptive immune responses (Hill, 1995; Guleria and Sayegh 2007; Mold et al., 2008). These include, expression of non-classical MHC molecules by trophoblast cells (King et al., 2000; Ishitani et al., 2003; Hunt et al., 2005; Lightner et al., 2008), tryptophan catabolism by the enzyme IDO (Munn et al., 1998), T cell apoptosis (Hunt et al., 1997), the complement system, regulatory T cells (Tregs) and inhibitory costimulatory molecule programmed death ligand (PD-L1 (Guleria et al., 2005; Lightner et al., 2008; Mold et al., 2008). It was also suggested that dendritic cells have a potential role in reproductive immunology and chemokine decoy receptors (Borroni et al., 2008; Kammerer et al., 2008). The classic, highly polymorphic MHC loci has a minimal expression, immediately following implantation, but it is increased steadily in placental and extravillous cytotrophoblast tissues during pregnancy (Kurpisz et al., 1995). Restriction of polymorphic MHC gene expression to later stages of embryonic development is probably useful to limit maternal rejection of the fetus. The contribution of each mechanism and the potential interactions among the various pathways are just beginning to emerge (Guleria and Sayegh, 2007).

Ligeois (1983) hypothesized that chimerism has a role in tolerance induction and maintenance during pregnancy. This hypothesis, although never been tested directly, is consistent with historical and recent findings which connect
induction of donor specific tolerance in fetal with cell chimerism during pregnancy.

In 1945, Owen found that bovine fraternal twins shared for life the blood cells types of both calves. Based on earlier observations, indicating that bovine twins share a single placenta and blood circulation during fetal phase (Lillie, 1916), Owen conjectured that blood cells and their precursors (which he called "embryonal cells ancestral to the erythrocytes") move from one twin to the other in the uterus, allowing mixing of blood cells from both genotypes (Owen, 1945). Bovine fraternal twins frequently exhibit a complete lifelong mutual tolerance to each other's leukocyte and a temporary (up to 15 months) tolerance to each other's skin grafts (Anderson et al., 1951; Billingham et al., 1952; Stone et al., 1965, 1971; Emery and McCullagh 1960a, b). In 1954, Owen et al. (1954), further detected another remarkable acquired tolerance phenomenon, connected to embryonic exposure. They reported that rhesus D negative pregnant mothers are less likely to produce antibodies against rhesus D positive child, in cases where the grandmother is rhesus D positive. This observation led to the discovery that a high percent of individuals tolerate non-inherited maternal HLA antigens (NIMA), better than non-inherited paternal HLA antigens NIPA (Claas et al., 1988, 1989). Then, it was hypothesized that exposure of a child to NIMA during pregnancy may lead to NIMA-specific tolerance later in life. Chimerism has probably an important role in this acquired tolerance effect (Owen et al., 1954; Andressy et al., 2003; van den Boogaardt et al., 2006). Billingham et al. (1953) were the first to induce specific tolerance to solid organs by exposing fetal and neonatal mice to the donor hematopoietic cell infusions. Engrafted mice did not reject allogeneic material, accepting skin from mice of the leukocyte donor strain, but not from any other strain (6-8 weeks after birth; Billingham et al., 1953; Billingham and Brent, 1956). Tolerance to skin allograft was permanent or transient, depending on the mouse strain (Billingham and Brent, 1956). This acquired tolerance to donor tissues was associated with leukocyte chimerism, which was detected in the animals' lymphoid organs (Billingham et al., 1953; Billingham and Brent, 1956). Since then, there have been numerous reports suggesting that transfer of foreign antigens from the mother to the fetus is common (review in Adams and Nelson, 2004). Recently, Mold et al. (2008) found that substantial numbers of maternal cells cross the placenta to reside in fetal lymph nodes, inducing the development of regulatory T cells (Tregs) that suppress fetal anti-maternal immunity and persist at least until early adulthood (Mold et al., 2008). The above study reveals a form of antigen-specific tolerance in humans induced in utero via chimerism and highlights the potential important role of cell chimerism for induction of fetal tolerance to maternal tissues.

Colonial protochordates may offer a unique evolutionary perspective on the maternal-fetal relationship during pregnancy. In these organisms, a genetically controlled allore cognition system, similar to the natural killer missing self model system in vertebrate, enables the creation of chimeric state (through vasculature fusion) with kin and prevents chimerism with non-related individuals (Oka and Watanabe, 1957; Sabbadin, 1962; Grosberg, 1988; Buss, 1982, 1990; Scofield et al., 1982).

Protochordates, are considered as the closest invertebrate relative of vertebrate (Delsuc, 2006). Studying the immunobiology of the tolerance to partial allograft in these organisms may reveal the evolutionary precursors to the maternal-fetal relationship during pregnancy.

**Genetic basic for allograft acceptance or rejection in protochordate**

In colonial protochordate, like the *Botryllus schlosseri*, homeostasis is defined by generation of all organ systems every week. Colonies are initially formed by asexual reproduction of founder individuals, a product of sexual reproduction (review in Manni and Burighel, 2006; Manni et al., 2007). The progeny clone members are united under a single gelatinous tunic by a network of anastomosed extracorporeal blood vessels. Throughout adult life, the *B. schlosseri* generates its entire body every week. This cycle of development, includes the formation of all body organs including heart, respiration system, digestive system, and neural complex. Ovary and testis are formed within each individual when sexual reproduction commences (Burighel and Cloney, 1997; Manni and Burighel, 2006; Manni et al., 2007). In addition to their extraordinarily high developmental activity, allogeneic *Botryllus* colonies can fuse and create a chimera.

When co-specific *Botryllidae* colonies contact each other, several morphological and cellular allogeneic processes and reactions are developed, including: fusion, rejection, indifference (no reaction) and a temporary fusion followed by disconnection (see Fig 1 for detailed fusion process; Bancroft 1903, Oka and Watanabe 1957, 1960, 1967; Sabbadin 1962, 1982; Mukai 1967; Tanaka and Watanabe 1973; Scofield et al., 1982; Saito and Watanabe 1982, 1984; Scofield et al., 1983; Hirose et al., 1988; 1990; Boyd et al., 1990; Sabbadin et al., 1992; Rinkevich and Weissman 1992a, b; Rinkevich et al., 1994a, b, 1998; Saito et al., 1994; Chsdik-Furman and Weissman 1995; Ballarin et al., 1995, 1998, 2002; Cima et al., 2004, 2006; Rinkevich, 2005).

Fusion or non fusion response between adjacent colonies is determined by a single, highly polymorphic, fusibility/histocompatibility (Fu/HC) locus (Oka and Watanabe, 1957; Sabbadin, 1962; Scofield et al. 1982) which was isolated and characterized (De Tomaso et al., 2005). Colonies that share at least one allele in their Fu/HC locus would fuse upon contact, and would become a chimera, while those that don’t share any allele would not (Oka and Watanabe, 1957). Burnet (1971) suggested studying *Botryllus* as a model for the evolution of self-recognition. He wrote, "although self recognition in ascidians is not analogous to the immunological processes of vertebrates, it presents a primitive type of self and not self recognition from which adaptive immunity may have evolved" (Burnet,
Fig. 1 Fusion process between kin. Using time laps microscopy (ImagExpress as described in Voskoboynik et al., 2008), we followed and documented the fusion process between 6 different pairs of compatible B. schlosseri colonies. These observations revealed a more elaborate fusion process than the one described before. A) A mother colony and its embryo. B) Larva seeks for a settlement site near its mother colony. C) Initial contact is established between the tunics and ampullae of the mother colony and its offspring colony. D) Following partial fusion of the tunics the offspring ampullae 1 and 2 penetrate into the tunic of the mother colony. E) The offsprings’ penetrating ampullae change the shape of their tips into a cone like shape and through dynamic cycles of extension and retraction further penetrate and explore the tunic of the mother colony. F) 64 hours later, these 2 navigating ampullae fuse with the marginal blood vessel of the mother colony. G) Following partial fusion of the tunics, colony A ampulla number 3 penetrates into the tunic of its sibling colony B. H) Penetrating ampulla, of colony A changes its tip shape into a cone like shape and through dynamic cycles of extension and retraction further penetrates and explores the tunic of its sibling colony. I) 16 hours later, ampulla 3 fuses with the marginal blood vessel of its sibling colony. J) Only a navigator ampulla fuses and creates a common blood vessel. Once created, the number of common blood vessels in the chimera remains constant throughout the chimera life. K-L) 360 hours following ampullae penetration, colony B got resorbed by its sibling colony A. Amp, ampulla; H, hours following ampullae contact; bv, blood vessel; mbv, marginal blood vessel; ampullae and common blood vessels are outlined by a dotted line. Bar = 75 µm.
An extensive study of the Botryllid ascidians self-nonself recognition system in the last 25 years revealed that, while the colonial ascidian Fu/HC and the mammalian major histocompatibility complex (MHC) share phenomenological features, including polymorphism and specificity, their molecular structure is different. Similar to the MHC, the Fu/HC is highly polymorphic (Karakashian and Milkman 1967; Scofield et al., 1982; 1983; Grosberg and Quinn, 1986; Grosberg 1987; Rinkevich et al., 1995; Paz et al., 2003; De Tomaso et al., 2005; Ben-Shlomo et al., 2001, 2006, 2008). However, structural homologies were not found (Weissman et al., 1996; Rinkevich and Weissman, 1992b; Fagan and Weissman, 1997; Pancer et al., 1993, 1996a, b, c, 1997; Muller et al., 1994; Khalturin et al., 2003; De Tomaso et al., 2005; Nyholm et al., 2006). The Botryllus Fu/HC is not homologous to any molecules of the vertebrate MHC-based histocompatibility system. Moreover, the whole Botryllus Fu/HC locus does not have a syntenic region in the Clona genome or in the genomes of vertebrates (De Tomaso et al., 2005). Genes involved in adaptive immunity, which include the polymorphic MHC class I and II glycoproteins that present internal peptides to T cells, the clonally expressed T-cell receptors (TCRs), immunoglobulins (Igs) and the recombination activating genes (RAG1, RAG2), have not been identified in protochordates (Klein 1989, Laird et al., 2000; Dehal et al., 2002; Kaufman, 2002; Azumi et al., 2003; Khalturin et al., 2003; De Tomaso et al., 2005; Litman et al., 2005, 2007). In both mice and humans, the mediators of the adaptive immune system are thought to be responsible for the rejection of a transplant. The adaptive immune T cell system rejects grafts even if one of two alleles is shared, as T cells make an immune reaction against non-self allele gene products.

In contrast, allogeneic Botryllus colonies tolerate each other and create a chimera even if only one of the two Fu/HC alleles is shared. This is consistent with immune systems, like the NK system in vertebrates, wherein recognition of self prevents an immune reaction. Recently, NK cells have been recognized as active participants in the acute and chronic rejection of solid tissue grafts (reviewed in Kitchen et al., 2005). The importance of NK cell attributes has been first recognized in bone marrow transplantation, where NK cells are fully sufficient to reject hematopoietic cell transplants, even in the absence of T or B cell responses (lethally-irradiated or Lack MHC class I expression recipients; Hoglund et al., 1991; Bix et al., 1991; Manilay and Sykes, 1998). Recent studies suggest that NK contribute to organ rejection indirectly, by activating or helping effector cells, such as cytotoxic and helper T cells (reviewed in Kitchen et al., 2005). Studies also point to a potential involvement of NK in the induction of tolerance to solid graft in mix chimerism, a tolerance regimen that employs the generation of mixed hematopoietic chimerism through donor stem cell engraftment (Zhao et al., 2003). Another aspect that might connect mammalian NK and the Botryllus Fu/HC was recently raised by Lighter et al. (2008). Studying uterine NK, Lighter et al., pointed to a unique phenotype that uterine NK and the Botryllus Fu/HC might share. Uterine NK cells produce angiogenic growth factors and are potential regulators of decidual angiogenesis in early pregnancy (Ashkar and Croy, 2001; Hanna et al., 2006; Manaster and Mandelboim, 2008). They suggested that, as Botryllus Fu/HC is probably involved in the generation of a common vascular system between two individuals, uterine NK may share the same evolutionary roots as the Botryllus Fu/HC.

**Cell parasitism induces development of a foreign entity within host colony**

Inspired by the genetic control for allograft acceptance and creation of chimerism within kin in Botryllus, Burnet (1971) hypothesized the emergence of intraspecific parasitism along the evolution. Indeed, his hypothesis was later confirmed in Botryllus chimeras, where the replacement of host germline by a donor genotype was a syntenic (Sabbadin and Zaniolo, 1979). In a remarkable set of experiments Sabbadin and Zaniolo (1979) demonstrated this kind of parasitism in B. schlosseri. Years later Pancer et al. (1995), Stoner and Weissman (1996) and Stoner et al. (1999) confirmed these results and further showed that in a chimera, the blood, soma and germ cells, demonstrated the combine genotypes of both chimeric partners. Moreover, in many cases the circulating pluripotent cells of one partner parasitized either the soma or the germ line of the other partner and replaced the whole mass of gonads or the soma (bud/zooid) of several individuals in the host colony (termed gonads or somatic cell parasitism; G/SCP). In a few cases, a complete takeover of donor genotype occurred and the whole mass of gonads in the chimeric colony expressed solely the donor’s genotype (Sabbadin and Zaniolo 1979; Pancer et al., 1995; Stoner and Weissman 1996; Stoner et al., 1999). Under invariant environmental conditions, both germline and somatic cell parasitism followed repeatable hierarchies of “winner strains” and “loser strains” (Stoner et al., 1999). However, breeding experiments proved that only the hierarchical position of germ cell parasitism is sexually inherited (Stoner et al., 1999). The hierarchy of somatic parasitism in Botryllus chimeras is a plastic trait, as variations in the environmental conditions (such as seawater temperature) can be reversed; the winner – loser hierarchy at the somatic parasitism level (Rinkevich and Yankelevich, 2004). Botryllus colonies propagate a-sexually through budding, therefore somatic stem cell parasitism in host colonies can induce the development of partial allogeneic entities (buds) within the host colony. As a result, chimerism in protochordate could serve as a state that enables the development of a “virtual embryo” within the host colony (Voskoboynik et al., in press; Fig. 2).

Beside cell parasitism chimerism may alter tolerance and intolerance state in the colonies. Chimeras might fuse with colonies that they used to reject (on their non chimeric phase) or reject colonies that they used to fuse with (Mukai 1967; Sabbadin and Astorri, 1988). Moreover, in some cases, chimeric colonies will simultaneously fuse
Fig. 2 Virtual pregnancy: a development of a semi allogeneic entity in a host colony through asexual budding in a chimera of a mother colony and its offspring. Botryllus colonies propagate asexually through budding, therefore, somatic stem cell parasitism in host colonies can induce the development of a partial allogeneic entities (buds) within the host colony. In this illustration, an offspring colony (red) is fused with its mother colony (blue). Genetic analysis of the chimera’s buds and gonads can reveal several cell chimerism patterns. Either both genotypes are detected or only one genotype is detected. bv, blood vessel; Mbv, marginal blood vessel; red, offspring genotype; blue, mother genotype.

These studies show that the genetically controlled ability of Botryllus colonies to tolerate or reject other colonies can be altered by chimerism. The temporal and spatial dynamic of the chimeric cells, patterns of host/donor cells competition, niche occupation and immunoregulatory mechanisms for routing, timing and frequencies of chimeric cells have probably important role in the induction of tolerance or intolerance.

**Stem cells mediated chimerism**

The long-term ability of cells from one genotype to replace the germline and somatic cells of the host, led to hypothesize that cell parasitism in the chimeras is mediated by stem cells (Sabbadin and Zaniolo, 1979; Rinkevich and Weissman, 1987; Pancer et al., 1995; Stoner and Weissman 1996; Stoner et al., 1999). By transplanting a single cell, which expresses high enzymatic activity of aldehyde dehydrogenase and a set of serial engraftment assays, Laird et al. (2005) revealed that multipotent stem cells are responsible for a stable long-term chimerism in adult B. schlosseri colonies. Yet, the location of these cells remained unknown (Laird et al., 2005). Recently, by using cell engraftments, chimeric fusion techniques, and in vivo cell tracking by automated time lapse fluorescence microscopy,
endostyle niche (Voskoboynik et al., 2008). The endostyle produces thyroid hormones and serotonin and expresses several variety of factors that are involved in development and stem cell regulation like Wnt, Hox1, Pax 2/5/8, PL10, Cadherin, Raldh and PCNA (Canestro et al., 2008; Dunn, 1980; Hiruta et al., 2005; Nilsson et al., 1988; Pennati et al., 2001; Rosner et al., 2006; Rosner et al., 2007; Voskoboynik et al., 2008). The endostyle niche is the first somatic stem cell niche ever described in a protochordate and is one of the most accessible stem cell niches for in vivo studies. The discovery of a major stem cell niche in an organism with Fu/HC controlled chimerism, pluripotent stem cell parasitism, and tolerance or intolerance induction via chimerism promotes the Botryllus as an evolutionary model for studying molecular regulations of tolerance induction by cellular chimerism in the absence of an adaptive immune system.

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