Chapter 17
Functions of Vitellogenin in Eggs

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Abstract Our understanding of the functions of vitellogenin (Vtg) in reproduction has undergone an evolutionary transformation over the past decade. Primarily, Vtg was regarded as a female-specific reproductive protein, which is cleaved into yolk proteins including phosvitin (Pv) and lipovitellin (Lv), stored in eggs, providing the nutrients for early embryos. Recently, Vtg has been shown to be an immunocomponent factor capable of protecting the host against the attack by microbes including bacteria and viruses. Moreover, Pv and Lv that both are proteolytically cleaved products of maternal Vtg, as well as Pv-derived small peptides, all display an antibacterial role in developing embryos. In addition, both Vtg and yolk protein Pv possess antioxidant activity capable of protecting cells from damage by free radicals. Collectively, these data indicate that Vtg, in addition to being involved in yolk protein formation, also plays non-nutritional roles via functioning as immune-relevant molecules and antioxidant reagents.

17.1 Introduction

Nonplacental or non-trophotenic vertebrates and nearly all invertebrates are oviparous, with their eggs being fertilized externally (Jalabert 2005). Egg, or a haploid reproductive cell, which develops into a viable embryo after fertilization, is the final product of oocyte growth and differentiation (Lubzens et al. 2010). Generally, several steps are involved in egg development: formation of primordial germ cells (PGCs) and transformation of PGCs into oogonia and then into oocytes. Oocyte growth, particularly in oviparous species, is characterized by deposition of massive amount of maternal information and molecules needed for early embryo development, including RNAs, proteins, lipids, vitamins, and hormones (Lubzens et al. 2010; Patiño and Sullivan 2002). One of the most important proteins deposited in oocytes is vitellogenin (Vtg), a large precursor of the major yolk proteins (Yps).

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Vitellogenin (from latin *vitellus*, yolk, and *gener*, to produce) was first proposed over 48 years ago, by Pan et al. (1969), to describe the female-specific hemolymph protein precursor of egg yolk in insects. This term was later adopted for the yolk protein precursor of other egg-laying animals, regardless of its amino acid sequence and structure, and is currently widely used by the scientific community. Vtg is now known as a high molecular mass glycolipophosphoprotein circulating in the blood (vertebrates) or hemolymph (invertebrates) as a homodimer. In oviparous vertebrates, Vtgs are usually synthesized in the liver, mainly under estrogen (E2) control (Rosanova et al. 2002; Wallace 1985). Similarly, in invertebrates Vtgs are also synthesized in an extra-ovarian tissue such as hepatopancreas (in crustaceans) and the fat body (in insects; Girish et al. 2014; Mak et al. 2005; Meusy 1980; Tufail and Takeda 2008). Soon after synthesis, Vtgs are posttranslationally glycosylated and phosphorylated in the endoplasmic reticulum (ER) and Golgi complex before being tagged for export, secreted as homodimeric lipoprotein complexes, to the blood or circulating body fluid, and transported to the ovary, where they are internalized during vitellogenesis into growing oocytes via receptor (clathrin)-mediated endocytosis (Amano et al. 2008; Conner and Schmid 2003; Kolarevic et al. 2008; Sawaguchi et al. 2005; Wallace and Selman 1990; Williams et al. 2014).

Vtgs are multidomain proteins, belonging to the large lipid transfer protein (LLTP) superfamily. They typically contain three conserved domains, the LPD_N (also known as vitellogenin_N or LLT domain) at the N-terminus, the DUF1943 domain with unknown function, and the von Willebrand factor type D domain (vWD) at the C-terminus. Occasionally, a DUF1944 domain with unknown function is also present in between DUF1943 and vWD in some Vtg proteins from vertebrates such as chicken and fish (Hayward et al. 2010). Beginning at the N-terminus, a complete Vtg consists of a signal peptide, a lipovitellin (Lv) heavy chain (LvH), a phosphorylated serine-rich phosvitin (Pv), a lipovitellin light chain (LvL), and two Cys-rich C-terminal coding regions (β′ and CT) that are homologous to the von Willebrand factor type D domain in mammals. The Pv region can be absent, as observed in zebrafish Vtg3 and most invertebrate Vtgs.

Vtg, as an egg yolk protein precursor, is present in the females of nearly all oviparous species including fish, amphibians, reptiles, birds, most invertebrates, and the platypus and thus was once regarded as a female-specific protein; however, its synthesis, albeit in smaller quantities, has been shown to occur in male and even sexually immature animals (Engelmann 1979; Piulachs et al. 2003). This suggests that Vtg presumably fulfills a more general role independent of a gender. Initially, Vtg was considered to be the energy source for the developing embryos. However, our understanding of the function of Vtg in reproduction has undergone a transformation over the past decade. A series of studies have demonstrated several non-nutritional roles of Vtg and its derivatives Yps such as Pv and Lv. For example, the multiplicity or loss of *vtg* family in vertebrates has been shown to have broad implications for the mode of reproduction (Babin et al. 2007; Brawand et al. 2008; Finn et al. 2009). The differential expression and deposition of Vtgs in the growing oocytes have been shown to be able to determine the

cleavage pattern (Finn et al. 2009; Gilbert 2006). Likewise, the variable degradation of Vtg derivatives Yps during oocyte hydration, which makes the pelagic eggs buoyant, has been shown to be able to determine the egg type (Carnevali et al. 2001; Finn et al. 2002; Matsubara et al. 1999; Reith et al. 2001; Selman et al. 2001). Vtgs have also been shown to be a maternal immune-relevant factor in early embryos. In addition, Vtgs display antioxidant activity. In this chapter, we will mainly focus on the multifaceted functions of Vtgs in eggs and early embryos, especially of fishes.

17.2 Vtgs and Yps Are Determinants of Mode of Reproduction and Type of Eggs

The multiplicity or loss of *vtg* family in vertebrates has been argued to have broad implications for the type of the egg (pelagic or benthic), mode of reproduction (placental or nonplacental), and cleavage pattern (meroblastic or holoblastic). Vtgs are usually encoded by a multigene family in animals, and thus there are several isoforms of Vtg in a given species. For instance, six *vtg* genes have been identified in nematode (Blumenthal et al. 1984), four in *Xenopus* (Germond et al. 1984; Wahli et al. 1979), three in chicken (Schip et al. 1987; Silva et al. 1989), and eight in zebrafish (Wang et al. 2000, 2005). Vtg genes act in a dosage-dependent fashion, so that a correlation between gene copy number and speed of yolking can be observed. For example, fishes tend to possess multiple *vtg* gene copies and produce a larger amount of eggs in a shorter time than birds and reptiles (Buisine et al. 2002). It appears that the lineage-specific *vtg* gene duplications frequently determine the nature of fish eggs. There are primarily two types of eggs in fish: benthic and pelagic. It was shown that the differential expression of non-neofunctionalized and neofunctionalized *vtg* genes in *Acanthomorpha* teleosts is correlated with the benthic or pelagic type of spawned eggs (Finn et al. 2009).

The amount of Vtg and its derivatives Yps is also correlated with the cleavage symmetry and pattern. In general, yolk consisting of Yps inhibits cleavage. In eggs with relatively little Yps, i.e., isolecithal and mesolecithal eggs, cleavage is holoblastic, meaning that the cleavage furrow extends through the entire egg. Eggs containing large accumulations of Yps undergo meroblastic cleavage, wherein only a part of the cytoplasm is cleaved.

Yps are an evolutionary adaptation that enables an embryo to develop in the absence of an external food source. Eggs without large amount of Yps, such as sea urchin, usually develop into a feeding larva fairly rapidly. In mammals, around 30–70 million years ago (Mya), the ancestral Vtg-encoding genes were lost in all but the egg-laying monotremes, which have retained a single functional *vtg* gene. The loss of *vtg*-encoding genes in "placental" mammals is consistent with the gain of alternative nourishment resources, casein, for the mammalian offspring (Brawand et al. 2008). At the other extreme are the eggs of fishes, reptiles, and

birds, which are full of Yps. These animals develop without a larval stage because Yps in their eggs are sufficient to nourish embryo and support early development.

17.3 Vtgs and Yps Are Sources of Free Amino Acids for Eggs and Embryos

Nutritional reserves that are stored in egg yolk are crucial for the development of nonmammalian oviparous vertebrates and invertebrates (Wahli 1988). An individual eukaryotic cell may express more than 2500 proteins at any one time. This huge biodiversity of cellular proteins has also been confirmed for fish oocytes, where more than 600 different proteins have been identified during oogenic stages of zebrafish and gilthead seabream (Ziv et al. 2008), and 1379 discrete proteins have been identified in the ovary of zebrafish (Groh et al. 2011). Despite such impressive number of proteins in the developing oocytes of fish, the most abundant and major proteins in all fishes are Vtgs (Groh et al. 2011; Ziv et al. 2008), of which three functional forms are known, VtgAa, vtgAb, and VtgC. In oviparous species, the nutrients of embryos largely derive from Vtgs and Yps stored in eggs. In mammals, the nutritive lactation with complex milk, coupled with a sophisticated placenta, which evolved in parallel with eutherian viviparity, effectively rendered embryonic nourishment through Vtg completely dispensable (Brawand et al. 2008). In most teleosts, a complete type Vtg has a pentapartite structure encoded by two major genes, *vtgAa* and *vtgAb*, while a minor *vtgC* gene encodes a smaller dipartite protein (Finn 2007a; Finn and Kristoffersen 2007). The majority of *vtg* genes are expressed according to season and reproductive cycle, and their products are secreted into the circulating plasma from the liver (Babin et al. 2007; Mommsen and Korsgaard 2008; Wallaert and Babin 1994), although some genes (*vtgC*) may be transcribed in the intestine (Wang et al. 2005). This latter observation is reminiscent of *vtg* gene transcription in nematodes where six Vtgs are secreted into the body fluid from the intestine, and one of which (Vtg6) is then cleaved before being incorporated into the oocytes (Spieth and Blumenthal 1985). In teleost, and particularly in cold-water marine fish, the VtgC synthetized by the intestine may be involved in the transport of highly unsaturated fatty acids (HUFA) and polyunsaturated fatty acids (PUFA) from the gut to the ovary, because cold-water fishes have limited abilities to synthesize phospholipids (Tocher et al. 2008). Such essential HUFAs and PUFAs must be supplied in the maternal diet (Harel et al. 1994) and then be transported by lipoproteins to the growing oocytes in order to later meet the physiological needs of the developing embryos and larvae.

The physiological significance of Vtgs is usually attributed to its role in the transport of amino acids, lipids, phosphorous, and calcium to the egg. Once secreted into the circulating plasma, Vtgs together with their phospholipids and other diverse cargo are internalized by the oocyte via clathrin-mediated endocytosis (Fig. 17.1). Vtg internalization occurs in coated pits of oocyte plasma membrane; they rapidly

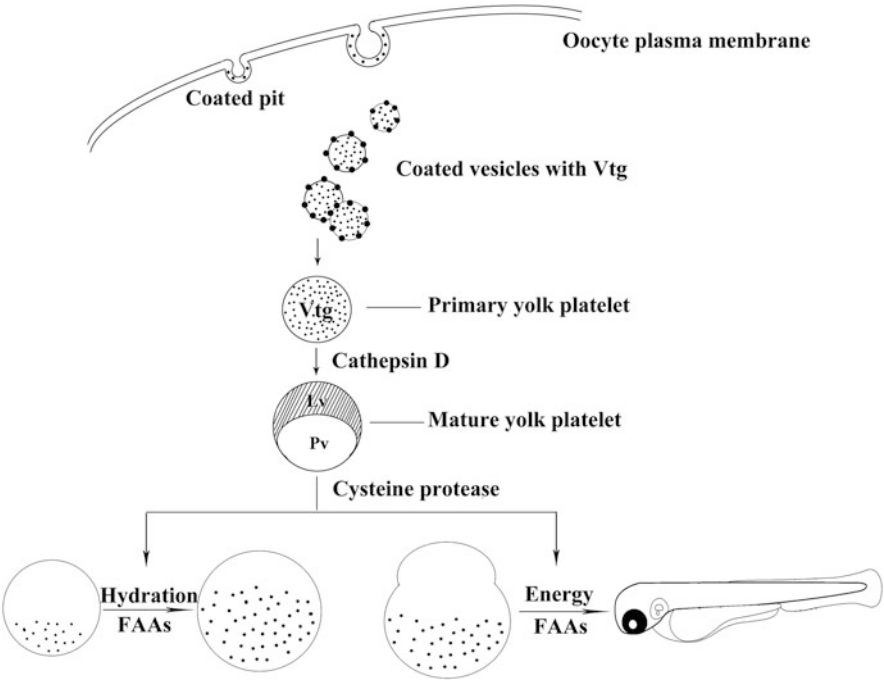

Fig. 17.1 Formation of yolk platelets from endocytosed Vtg and fate of Vtg-derived Yps Pv and Lv. Vtgs in the circulating plasma are taken in by the oocyte via clathrin-mediated endocytosis, forming coated vesicles in the cortical oocyte cytoplasm. The coated vesicles containing Vtg lose their clathrin coat and coalesce to form the primordial yolk globules, which are subsequently transformed into yolk platelets. Vtgs in the yolk platelets are primarily cleaved into Lv and Pv. In many marine fishes with pelagic eggs, an additional proteolysis of Yps occurs at the end of oocyte growth just before maturation, generating smaller peptides and free amino acids (FAAs), which form part of the osmotic gradient necessary for water uptake during the "hydration phase" that makes the pelagic eggs buoyant. During embryonic development, Lv and Pv are further cleaved into small peptides and FAAs, providing the nutrients for developing embryos

detach and give rise to coated vesicles in the cortical oocyte cytoplasm; the latter rapidly lose their clathrin coat and coalesce to form the primordial yolk globules subsequently transformed into yolk platelets (Ghiara et al. 1968; Limatola and Filosa 1989; Neaves 1972; Wallace 1985). Once packed in yolk platelets, Vtgs will undergo primary degradation. During degradation, Vtgs are cleaved by an aspartic proteinase called cathepsin D, into lipovitellins (Lv), phosvitin (Pv), β-component (β-C), and C-terminal coding region (CT) of different number and size (Komazaki and Hiruma 1999; Retzek et al. 1992). The Vtg-degraded products have different roles in oocytes and developing embryos. Lv, the largest yolk protein derived from the proteolytic processing of Vtgs, is an apoprotein delivering mainly phospholipids into developing oocytes (Romano et al. 2004; Yilmaz et al. 2015). Pv, the smallest yolk protein derived from the proteolytic process, largely consists of phosphorylated serine residues and is thought to be able to stabilize nascent Vtg

structure during lipid loading and to enhance solubility of Vtgs in the blood (Finn 2007b; Yilmaz et al. 2015). β-C and CT, the small cleavage products of vWD that contains a highly conserved motif of repeated cysteine residues, are postulated to stabilize the Vtg dimer for cellular recognition and receptor binding and to protect Vtg or its product Yps from premature or inappropriate proteolysis (Finn 2007b; Reading et al. 2009; Williams et al. 2014). In many marine fishes with pelagic eggs, an additional proteolysis of Yps occurs at the end of oocyte growth just before maturation. This proteolysis generates smaller peptides and free amino acids (FAAs), which form part of the osmotic gradient necessary for water uptake during the "hydration phase" that makes the pelagic eggs buoyant (Finn and Fyhn 2010). During embryonic development, a different enzymatic pool, reported to be cysteine proteases, causes the secondary degradation of both Lv and Pv. This secondary cleavage of Yps generates a supply of small peptides and FAAs, which are important for developing embryos. The earliest stage of fish embryos, particularly before the mid-blastula transition, does not synthetize their own protein and is entirely dependent on the provision of maternal nutrients and transcripts or signaling factor that are produced during oogenesis. Therefore, the Vtgs-degraded FAAs are the essential energy supply for developing embryos.

Recently, Pv has been shown to be involved in bone formation in chicken embryos. In cultured calvarial osteoblasts, Pv is capable of stimulating the differentiation of osteoblasts, collagen synthesis, hydroxyproline formation, and biomineralization. Moreover, Li et al. (2014) showed that lysophophatidic acid, a signaling molecule derived from phosphatidylcholines of Pv (Ohlendorf et al. 1977), is able to regulate hemangioblast formation and primitive hematopoiesis in zebrafish. These data suggest that Pv and the smaller molecules derived from Pv also play important roles during embryonic development.

17.4 Vtgs and Yps Are Maternal Immune-Relevant Factors in Eggs and Embryos

Embryos of most mammalian species including humans develop in the uterus inside the mother's body and are thus well protected from external pathogenic attacks. In sharp contrast, eggs of most fish and aquatic invertebrates are released and fertilized externally, and therefore the resulting embryos are exposed to a hostile aquatic environment full of potential pathogens, which are capable of causing various types of diseases, even leading to death. For instance, it was shown that exposure of salmon fry and juveniles to the Gram-negative bacterium *Yerinia ruckeri* caused occurrence of enteric redmouth disease, resulting in 60% mortality (Haig et al. 2011). In addition, during the early stages of development, their embryos have little or limited ability to synthesize immune-relevant molecules endogenously, and their immune-relevant cells and tissues are not yet fully formed (Ellis 1988; Liang et al. 2009; Magnadóttir et al. 2004). How the embryos of fish and aquatic invertebrates

survive the pathogenic attacks at this stage has received great attention in the past two decades. It is well known that fishes and aquatic invertebrates produce eggs endowed with all the nutrients and protective systems allowing the development of a fish embryo in an aquatic environment. It has been shown that embryo protection is partly ensured by Vtgs and their derived proteins Yps.

Accumulating data show that Vtgs are associated with antibacterial activity of the host against microbes including bacteria and virus (Garcia et al. 2010; Shi et al. 2006; Zhang et al. 2011). In zebrafish, Vtg has been shown to function as an acute phase protein in vivo, capable of leading to elimination of invading bacteria such as *Escherichia coli* and *Staphylococcus aureus* (Tong et al. 2010). Shi et al. (2006) showed that intraperitoneal injection of *E. coli* was able to enhance the level of serum Vtg in male rosy barb *Puntius conchonius*; and Lu et al. (2012, 2013) showed that the challenge with Gram-negative bacterium *Citrobacter freundii* induced upregulated expression of *vtg* in the skin of zebrafish. Bacterial challenge also caused a significant increase in *vtg* expression in scallop and insect (Nachappa et al. 2012; Wu et al. 2015). All these data suggest that Vtgs play an active role in protecting the host from infection. There is solid evidence that Vtgs are involved in the antimicrobial defense of the host against broad-spectrum bacteria and virus. Vtgs have been shown to act as a multivalent pattern recognition receptor capable of binding to lipopolysaccharide, lipoteichoic acid, peptidoglycan, glucan, and virons, a bactericidal molecule capable of damaging bacterial cell walls, and an opsonin capable of enhancing the phagocytosis of bacteria by macrophages (Garcia et al. 2010; Li et al. 2008; Sun and Zhang 2015; Zhang et al. 2011, 2015). Notably, Vtgs are shown to remain uncleaved in the oocytes of amphioxus and nematode (Sharrock 1983; Sun and Zhang 2001). Thus, it is possible that Vtgs in these animals may protect their oocytes and embryos against pathogenic attack.

Lv and Pv are the major Yps generated by the proteolytic cleavage of Vtg. As parental Vtg is an immune-competent molecule, it is thus reasonable to speculate that Lv and Pv also have similar immune activities. In fact, Pv has been proven to play a critical role in the immunity of zebrafish embryos via acting as a pattern recognition receptor and an antimicrobial effector molecule (Wang et al. 2011). Also smaller peptides derived from Pv have antibacterial activity (Wang et al. 2011; Zhang et al. 2015). In line with this, hen egg yolk Pv was also shown to be able to inhibit the growth of the Gram-negative *E. coli* and the Gram-positive bacterium *S. aureus* under thermal stress (Sattar Khan et al. 2000; Ma et al. 2013). Moreover, Pv from zebrafish was demonstrated to possess antiviral activity via inhibiting the formation of the cytopathic effect in lymphocystis disease virus-infected cells and reducing the virus quantities in the virus-infected cells and host. This indicates that Pv is a maternal immune-relevant factor capable of protecting developing embryos from virus attack (Sun et al. 2013). Like Pv, both LvH and LvL are also maternal factors capable of protecting early embryos and larvae (Liang et al. 2016; Zhang et al. 2011). These show that Pv and Lv are both maternally derived proteins involved in immune defense in embryos and larvae in fishes.

17.5 Vtgs and Yps Are Antioxidant Reagents in Eggs and Embryos

Oxidation is a chemical reaction that can produce free radicals, resulting in chain reactions that can cause extensive damage to DNA, proteins, and lipids. Therefore, antioxidant defense is thought to be important throughout an organism's life. This is also the case during embryo development and growth because intense embryonic metabolism entails massive production of oxidizing molecules. How rapidly growing embryos protect themselves from damage by free radicals is an interesting question in ecological evolutionary studies and in animal production disciplines (Ebrahimi et al. 2012; Müller et al. 2012; Romano et al. 2008; Saino et al. 2002, 2003; Selim et al. 2012; Surai 2002). It has been shown that the eggs of oviparous animals contain large amounts of antioxidants of maternal origin (Surai 2002). Mothers equip their eggs with various antioxidants such as vitamin A, vitamin E, and β-carotene (Barim-Oz and Sahin 2016; Dale et al. 2017). Among them, the specific antioxidants present in egg yolk are important for embryonic development (Ayala et al. 2006; Gao et al. 2013; Romano et al. 2008; Saino et al. 2003).

Another unique role of Vtgs is antioxidant/reducing activity. It was first shown that Vtg from eel (*Anguilla japonica*) was able to resist the copper-induced oxidation and seemed to chelate low concentrations of copper ion and could protect the very low-density lipoprotein (VLDL) against copper-induced oxidation (Ando and Yanagida 1999). This was the first report that Vtg is antioxidant reagent and serves to depress the free-radical reactions in fish oocytes. Later, it was demonstrated that Vtg has a role to protect other cellular components from oxidation because of its metal binding capacity in the nematode *C. elegans* (Nakamura et al. 1999). In the honeybee, Vtg was also shown to be able to reduce oxidative stress by scavenging free radicals, thus increasing the life span of the sterile honeybee workers and queen honeybee. The honeybee Vtg was also capable of recognizing cell damage through membrane binding and shielding living cells from reactive oxygen species (Havukainen et al. 2013). It is clear that Vtg can protect cells from free radical damage in both invertebrates and vertebrates.

Like Vtgs, the yolk protein Pv also shows antioxidant ability. It is well known that hen Pv serves as an antioxidant to inhibit metal-catalyzed phospholipid oxidations and hydroxyl radical formation (Guérin-Dubiard et al. 2002; Ishikawa et al. 2004; Lu and Baker 1986). Similarly, zebrafish recombinant Pv was also shown to be an antioxidant agent capable of inhibiting the oxidation of the linoleic acid and scavenging the DPPH radical (Hu et al. 2015). It is possible that antioxidant function of Pv can protect rapidly developing embryos from damage by free radicals. It is worthwhile to explore if other components derived from Vtgs such as Lv, β-C, and CT have antioxidant activity.

17.6 Conclusions

Our understanding of the functions of Vtgs in animal reproduction has undergone a revolutionary transformation over the past decade. This chapter summarized the state-of-the-art knowledge of Vtgs in animal reproduction. Primarily, Vtg was regarded as a female-specific reproductive protein, which is cleaved into Yps such as Pv and Lv, stored in eggs, providing the nutrients for developing embryos. However, recently, the Vtg was also shown to be an immune-relevant molecule involved in the defense of the host against the microbes including bacteria and viruses. Furthermore, Pv and Lv that both are proteolytically cleaved products of maternal Vtg, as well as Pv-derived small peptides, display an antibacterial activity in developing embryos. Finally, both Vtg and yolk protein Pv are an antioxidant reagents capable of protecting cells from damage by free radicals.

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