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REVIEW

Adipogenesis in fish

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ABSTRACT

White adipose tissue (AT) is the main lipid storage depot in vertebrates. Initially considered to be a simple lipid store, AT has recently been recognized as playing a role as an endocrine organ that is implicated in processes such as energy homeostasis and as a rich source of stem cells. Interest in adipogenesis has increased not only because of the prevalence of obesity, metabolic syndrome and type 2 diabetes in humans, but also in aquaculture because of the excessive fat deposition experienced in some cultured fish species, which may compromise both their welfare and their final product quality. Adipocyte development is well conserved among vertebrates, and this conservation has facilitated the rapid characterization of several adipogenesis models in fish. This Review presents the main findings of adipogenesis research based in primary cultures of the preadipocytes of farmed fish species. Zebrafish has emerged as an excellent model for studying the early stages of adipocyte fish development *in vivo*. Nevertheless, larger fish species are more suitable for the isolation of preadipocytes from visceral AT and for studies in which preadipocytes are differentiated *in vitro* to form mature adipocytes. Differentiated adipocytes contain lipid droplets and express adipocyte marker genes such as those encoding the peroxisome proliferator activated receptor γ (*pparγ*), CCAAT-enhancer-binding protein α (*c/ebpα*), lipoprotein lipase (*lpl*), fatty acid synthase (*fas*), fatty acid binding protein 11 (*fabp11*), fatty acid transporter protein1 (*fatp1*), adiponectin and leptin. Differentiated adipocytes also have elevated glycerol 3-phosphate (G3P) dehydrogenase (GPDH) activity. To better understand fish adipocyte development and regulation, different adipokines, fatty acids, growth factors and PPAR agonists have been studied, providing relevant insights into which factors affect these processes and counterbalance AT dysregulation.

KEY WORDS: Lipid metabolism, Aquaculture, Adipocyte, Cell culture, Adipose tissue

Introduction

A global interest in white adipose tissue (AT), commonly known as fat, has increased during recent decades because of the increased prevalence of obesity, which enhances the risk of developing cardiovascular diseases and type 2 diabetes in humans (Lavie et al., 2009; Nath et al., 2006). In fish, the interest in AT has been stimulated by two main factors: (1) a desire to control the excess of fat accumulation of some fish species in aquaculture, and (2) the potential use of zebrafish (*Danio rerio* Hamilton) as an *in vivo* vertebrate model to study human obesity and its associated disorders. AT homeostasis relies on the proper function of its adipose stromal vascular fraction (aSVF) cell population and on the maintenance of the number and/or size of its adipocytes, and these requirements apply to all vertebrates. AT grows by increasing the volume of its existing adipocytes (hypertrophy) and/or by increasing their number through the differentiation of precursor cells (adipose hyperplasia or adipogenesis). Previous studies in fish AT unraveled the existence of a broad adipocyte size distribution *in vivo* (Weil et al., 2013), confirming that AT in fish (as in other vertebrates) expands by both hypertrophic and hyperplastic growth. In humans, adipogenesis has been referred to as a 'healthy' expansion of AT (Choe et al., 2016; Rutkowski et al., 2015), whereas hypertrophy and a limited adipogenic capacity are linked to metabolic disorders (e.g. those in obesity) (Choe et al., 2016). AT homeostasis is dysregulated during excessive hypertrophy, leading to: (i) improper secretion of AT bioactive molecules or adipokines (e.g. a decrease in adiponectin and an increase in pro-inflammatory cytokines); (ii) an increase in immune cell recruitment, hypoxia, fibrosis and metabolites [e.g. an increase in lipolysis or basal free fatty acid (FA) release] and (iii) impaired insulin sensitivity leading to metabolic stresses and metabolic disorders (Choe et al., 2016). In fish, some nutrients that are currently used for fish feed, such as vegetable oils, induce more hypertrophic AT growth, lipolysis and lipid accumulation than do fish oils, provoking an excess of fat deposition that decreases the product quality (Cruz-Garcia et al., 2011; Todorčević et al., 2008). Therefore, both biomedicine and aquaculture research are trying to unravel which physiological and molecular mechanisms regulate AT hypertrophy and hyperplasia in vertebrates in order to control them.

Unfortunately, there are currently no fish cell lines available for the study of adipogenesis; therefore, several primary cell cultures of the aSVF (also known as preadipocyte cultures) have been established in different fish species that are of interest to aquaculture, and these will be discussed in detail in this Review. In contrast to these fish *in vitro* cultures, in which cells are grown under controlled conditions outside the body, *in vivo* models such as zebrafish reflect the precise environment where adipogenesis occurs. Zebrafish have proved to be a great model for studying human diseases that are associated with excessive adipogenesis because zebrafish and humans share key conserved organs (e.g. pancreas, AT, liver and muscle) that are important for energy homeostasis and metabolism, as well as similar hypothalamic appetite circuits, insulin regulation, endocrine signaling through leptin, and lipid storage in adipocytes (Den Broeder et al., 2015). Adipogenesis in zebrafish has been broadly reviewed in recent years (Den Broeder et al., 2015; Seth et al., 2013). The present Review focuses mostly on studies of adipogenesis in non-zebrafish fish species.

To date, there is no evidence of the existence of brown or beige adipocytes in teleost fish; therefore, this Review focuses on the development of white adipocytes, referred to as adipocytes from this point onwards. In this Review, I first describe the biology of the AT in fish from its possible origins to the synthesis of intracellular lipids in adipocytes. Second, I discuss the mechanisms involved in fish AT growth (hypertrophy and hyperplasia), emphasizing the two

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main phases of adipogenesis (determination and differentiation). I subsequently review the *in vitro* models used to study fish adipogenesis, as well as their molecular profiles during adipocyte differentiation and their regulation by adipokines, growth factors, FAs and other molecules. Finally, I summarize the main findings of recent studies in relation to adipogenesis in fish.

Biology of adipose tissue in fish

AT is a specialized type of connective tissue filled with neutral lipid-filled cells called adipocytes. AT participates in whole-organism energy homeostasis through: (i) regulating the cellular lipid turnover of its adipocytes and (ii) the secretion of adipokines [e.g. adiponectin, leptin and tumor necrosis factor α (TNFα)], which have an endocrine function and act on the central nervous system (CNS) and peripheral tissues to regulate processes such as appetite, glucose homeostasis and lipid metabolism (Esteve Ràfols, 2014; Konige et al., 2014; Rutkowski et al., 2015; Salmerón et al., 2015a, b; Saltiel, 2001; Sánchez-Gurmaches et al., 2012). Teleost fish accumulate lipids, mainly in the form of triglycerides (TG), in different anatomical sites (visceral organs, liver, subcutaneous tissues, red and white muscles, brain, pancreas, esophagus, mandible, cranium and tail fin) depending on the species, the nutritional state, the life-stage and the physiological state (Flynn et al., 2009; He et al., 2015; Weil et al., 2013). The perivisceral fat, subcutaneous fat, muscle and liver have been described as the preferential lipid storage sites in teleost fish (Flynn et al., 2009; He et al., 2015; Weil et al., 2013). Furthermore, fish AT participates actively as a source of metabolic energy for growth, reproduction, embryonic and yolk-sac larval development and swimming (Tocher, 2003). In zebrafish, the first adipocytes appear in a right-sided visceral position in close proximity to the pancreas after 8 days post fertilization (Flynn et al., 2009). They rapidly increase in number and in the size of their neutral lipid droplets (LD) and expand their anatomical distribution; however, they are mainly concentrated in the visceral cavity in adult fish (Flynn et al., 2009). Zebrafish adipogenesis requires and is regulated by exogenous nutrition: the adipocytes remodel their lipid storage as a function of nutrient availability (Flynn et al., 2009).

Possible origins of the adipocytes in fish

In vertebrates, AT contains not only adipocytes but also an aSVF formed by a heterogeneous population of cells such as preadipocytes, pericytes, endothelial cells, smooth muscle cells, fibroblasts, neuronal cells, hematopoietic-lineage cells and mesenchymal stem cells [MSCs, also called adipose tissue-derived stem cells (ASCs)] (Bourin et al., 2013; Cawthorn et al., 2012; Todorčević et al., 2010). ASCs are multipotent cells that have the ability to differentiate into adipocytes, chondrocytes, osteoblasts or myocytes, among other cell lineages (Cawthorn et al., 2012). Recent evidence from mammals supports the view that the ASCs originate from the perivascular or endothelial region of AT (the pericytes), as well as from other non-adipose sources of progenitor cells such as bone-marrow-derived hematopoietic stem cells (Hilton et al., 2015). Two studies, one in Atlantic salmon (*Salmo salar* L.) (Todorčević et al., 2010) and the other in zebrafish (Flynn et al., 2009), have identified the potential origins of the adipocyte lineage in fish. Todorčević and collaborators detected a heterogenous cell population in the early stages of a primary culture of aSVF cells from Atlantic salmon, with cells expressing transcriptional markers for vascular cells, macrophages, lymphocytes and preadipocytes (Todorčević et al., 2010) (Fig. 1A). The authors suggest that this early transcriptional profile implies the retention of multipotency of the aSVF cells; this hypothesis was supported recently by a study by Ytteborg and collaborators, in which the addition of an osteogenic medium produced the differentiation of preadipocytes to form mineralizing osteogenic cells (Ytteborg et al., 2015) (Fig. 1A). Interestingly, when Atlantic salmon adipocyte precursor cells reach confluency, they express transgelin, a marker of multipotent vascular pericytes, suggesting a potential perivascular origin of salmon (pre)adipocytes like that described in mammals (Tang et al., 2008; Todorčević et al., 2010) (Fig. 1A). In zebrafish, Flynn and collaborators propose two models for the origin of the visceral preadipocytes, which first appear in association with the pancreas shortly after the initiation of exogenous nutrition (Flynn et al., 2009). In the first model, the precursors are fatty acid binding protein 11a (fabp11a) (a homolog of mammalian Fabp4)-negative cells located in the pancreas and in

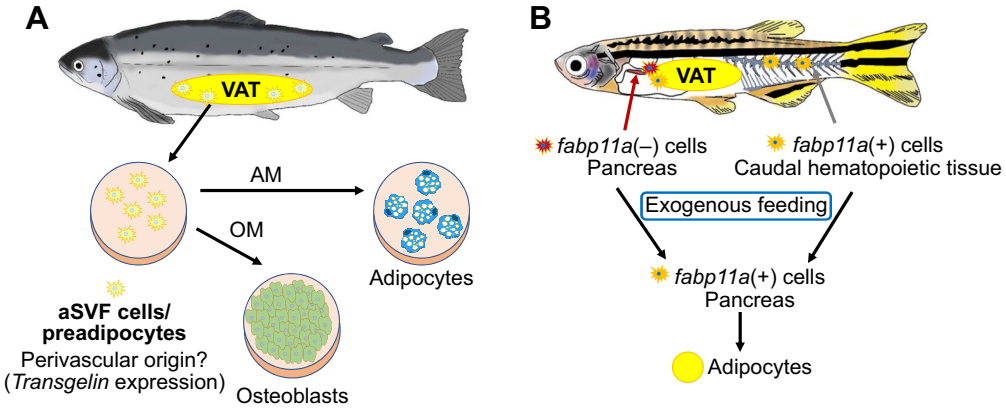

Fig. 1. Possible origins of visceral adipose tissue (VAT) adipocytes in fish. (A) During the early stages of the primary culture of adipose stromal vascular fraction (aSVF)–preadipocytes of Atlantic salmon, the cells express several transcriptional markers. These include *transgelin*, a marker of multipotent vascular pericytes, suggesting a potential perivascular origin of salmon adipocytes. The aSVF cells retain multipotency and they can be differentiated to adipocytes or osteoblasts depending on whether an adipogenic (AM) or an osteoblastogenic (OM) medium is used. (B) Zebrafish (pre)adipocytes first appear in association with the pancreas shortly after initiation of exogenous nutrition and they express *fatty acid binding protein 11a* (*fabp11a*) during adipogenesis. Two models have been proposed to explain the origin of these *fabp11a*-positive [*fabp11a*(+)] cells in the pancreas. In the first model, *fabp11a*-negative [*fabp11a*(–)] cells that are already present in the pancreas differentiate to become *fabp11a*(+) after exogenous feeding. The second model proposes that *fabp11a*(+) cells located in the caudal hematopoietic tissue respond to exogenous feeding by mobilizing into the circulating blood and then colonizing the pancreas where they differentiate to form adipocytes.

Journal of Experimental Biology (2018) 221, jeb161588. doi:10.1242/jeb.161588

the adjacent stroma or vasculature. After exogenous feeding, these cells differentiate into fabp11a-expressing visceral (pre)adipocytes (Fig. 1B). In the second model, fabp-11a-positive precursor cells are located in the caudal hematopoietic tissue (Fig. 1B). These cells respond to exogenous feeding by mobilizing into the circulating blood and then colonizing the pancreas where they differentiate (Flynn et al., 2009) (Fig. 1B). Therefore, teleost fish probably possess visceral AT that has an origin similar to that in other vertebrates.

Lipid metabolism in adipocytes

AT plays an important role in regulating body lipid metabolism for several reasons. First, mature adipocytes serve as energy reserves because (in a process called lipogenesis) they take FAs or other substrates (e.g. glucose or amino acids) from the diet and convert them into TG for long-term storage. Second, during periods of energy requirement, the fat cells break down these TG (in a process called lipolysis) into FAs and glycerol, which can then be released into the blood. A third way in which lipids are metabolized in the AT, although minor, is their oxidation to provide energy in the mitochondria. These processes of lipogenesis, lipolysis and β-oxidation are regulated by various hormones and growth factors, with the involvement of many enzymes and transcription factors. During adipocyte differentiation in fish, the expression and activity of these enzymes increases as part of the normal biochemical functions of the mature adipocytes.

TG are formed from glycerol 3-phosphate (G3P) and three FAs molecules, which originate from different metabolic pathways. The glycerol necessary for TG synthesis comes from three sources (Fig. 2): (i) glycolysis [glycerol-3-phosphate dehydrogenase (GPDH) uses nicotinamide adenine dinucleotide (NADH) to reduce dihydroxyacetone phosphate (DHAP) to G3P]; (ii) glyceroneogenesis [DHAP is synthesized from amino acids and tricarboxylic acid (TCA) cycle intermediates such as pyruvate and lactate]; or (iii) phosphorylation by glycerol kinase (GK) of the glycerol generated during lipolysis (Proença et al., 2014). Glyceroneogenesis is the dominant pathway for G3P synthesis *in vivo* in rats, contributing ∼90% of the G3P produced in epididymal and mesenteric AT (Nye et al., 2008). Cytoplasmic phosphoenolpyruvate carboxykinase (PEPCK-C), which catalyzes the decarboxylation of oxaloacetate (OAA) to form phosphoenolpyruvate (PEP), is the rate-limiting enzyme of the glyceroneogenic pathway (Fig. 2). After multiple conversion steps, the PEP formed by PEPCK-C is converted to 1,3-bisphosphoglycerate (1,3BPG). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) reduces 1,3BPG to glyceraldehyde-3-phosphate (GADP), which is then converted to DHAP by triose phosphate isomerase (TPI).

The composition of the three FAs that are required to form a TG can be different (e.g. in the number of carbons or in whether they are saturated or unsaturated). Adipocytes obtain these FAs from three main sources (Fig. 3): (i) incorporation of FAs bound to albumin; (ii) hydrolysis of lipoproteins (e.g. chylomicrons and very-low-density lipoproteins) by the lipoprotein lipase (LPL); or (iii) synthesis of FAs within the adipocyte from acetyl coenzyme-A (Proença et al., 2014). The FAs from the first two sources are carried by the blood. They cross the plasma membrane using FA transporters such as cluster of differentiation 36 (CD36), fatty acid transporter protein (FATP), and plasma membrane FABP (FABPm) (Fig. 3). Cytoplasmic FABP (FABPc), such as FABP-4, carry these FAs from the membrane to the localization of the acyl-CoA synthase to be esterified as acyl-CoA (Fig. 3). Finally, this acyl-CoA is carried by an acyl-CoA binding protein (Acyl-CoA-BP) to the endoplasmic reticulum to be esterified with G3P to form TG (Fig. 3). The third source of cellular FAs is the synthesis of acetyl-CoA and FAs from non-lipid precursors (e.g. carbohydrates) and is also known as *de novo* lipogenesis (DNL) (Proença et al., 2014). It has recently been shown that DNL is active in Atlantic salmon preadipocytes in culture; however, the conversion of the glucose into lipids makes a relatively small contribution to the production of cellular lipids for storage (less than 0.1%), and this limited capacity may contribute to glucose intolerance in salmonids (Bou et al., 2016). During DNL, acetyl-CoA is carboxylated by acetyl-coA carboxylase (ACC) to

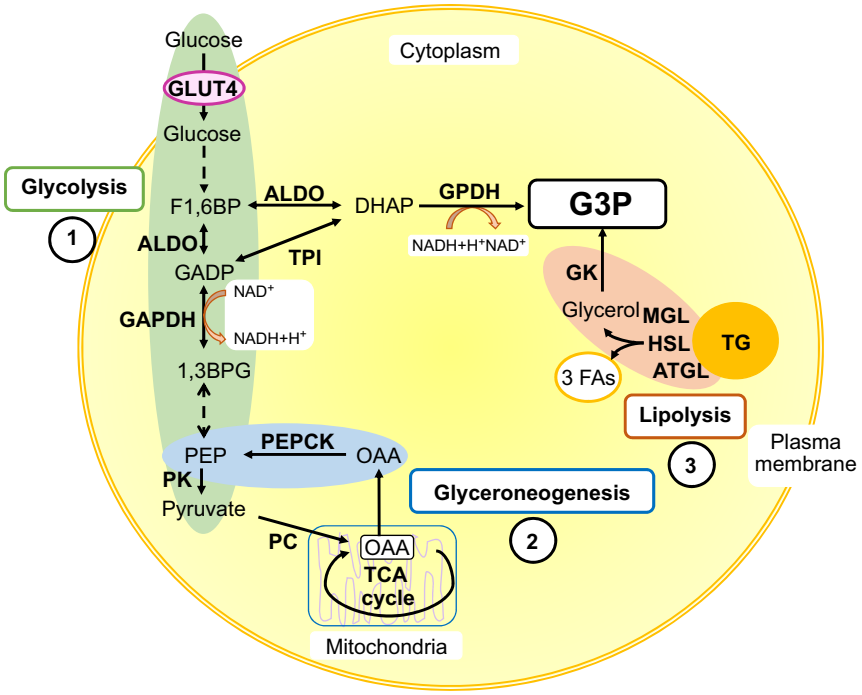

Fig. 2. Metabolic glycerol 3-phosphate sources used to produce triglycerides. Triglycerides (TG) are formed by a backbone of glycerol 3-phosphate (G3P), which can originate from three main sources: (1) glycolysis, (2) glyceroneogenesis or (3) from glycerol generated during lipolysis. Abbreviations: ALDO, fructose-bisphosphate aldolase; ATGL, adipose triglyceride lipase; 1,3BPG, 1,3-bisphosphoglycerate; DHAP, dihydroxyacetone phosphate; FA, fatty acid; F1,6BP, fructose 1,6-bisphosphate; GADP, glyceraldehyde 3-phosphate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GK, glycerol kinase; GLUT4, glucose transporter type 4; GPDH, glycerol-3-phosphate dehydrogenase; HSL, hormone-sensitive lipase; MGL, monoacylglycerol lipase; NAD^+/H, nicotinamide adenine dinucleotide oxidized and reduced, respectively; PC, pyruvate carboxylase; PEP, phosphoenolpyruvate; PEPCK, phosphoenolpyruvate carboxykinase; PK, pyruvate kinase; OAA, oxaloacetate; TCA cycle, tricarboxylic acid cycle; TPI, triose phosphate isomerase. Solid arrows indicate single-step enzymatic reactions, whereas dashed lines indicate multi-step reactions.

Journal of Experimental Biology (2018) 221, jeb161588. doi:10.1242/jeb.161588

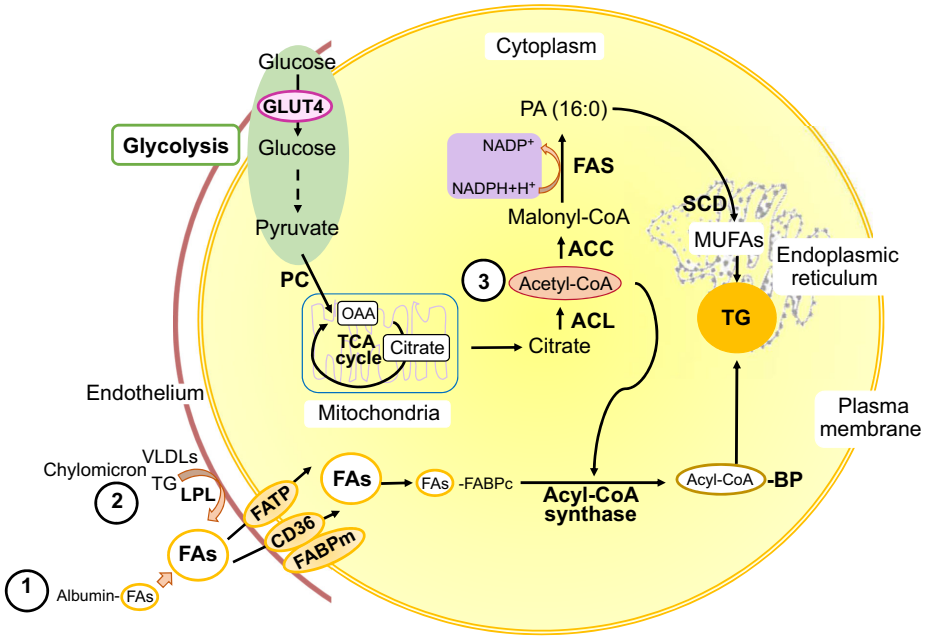

Fig. 3. Metabolic sources of fatty acids used to produce triglycerides. Triglycerides (TG) are formed by a backbone of glycerol 3-phosphate (G3P) and three fatty acid (FA) molecules that can originate from three main sources: (1) FAs bound to albumin; (2) hydrolysis of lipoproteins [e.g. chylomicrons and very-low-density lipoproteins (VLDLs)] by the lipoprotein lipase (LPL); or (3) synthesis within the adipocyte from acetyl coenzyme-A (CoA). Abbreviations: ACC, acetyl-CoA carboxylase; ACL, ATP-citrate lyase; acyl-CoA-BP, acyl-CoA binding protein; CD36, cluster of differentiation 36; FABPc, cytoplasmic fatty-acid-binding protein; FABPm, plasma membrane fatty-acid-binding protein; FAS, fatty acid synthase; FATP, fatty acid transport protein; GLUT4, glucose transporter type 4; MUFA, monounsaturated fatty acid; $NADP^+$/H, nicotinamide-adenine dinucleotide phosphate oxidized and reduced, respectively; OAA, oxaloacetate; PA (16:0), palmitic acid; PC, pyruvate carboxylase; TCA cycle, tricarboxylic acid cycle; SCD, stearoyl-CoA desaturase. Solid arrows indicate single-step enzymatic reactions, whereas dashed lines indicate multi-step reactions.

form malonyl-CoA (Fig. 3), and OAA is reduced by malate dehydrogenase (MDH) to form malate. Then, fatty acid synthase (FAS) assembles malonyl-CoA and acetyl-CoA using the cofactor reduced nicotinamide-adenine dinucleotide phosphate (NADPH) to form palmitic acid (PA) (16:0) (Fig. 3), which in turn can be elongated to form stearic acid (18:0). Stearoyl-CoA desaturase, the rate-limiting step in the synthesis of monounsaturated FAs, desaturates PA (16:0) and stearic acid (18:0) to form palmitoleic acid (16:1n-7) and oleic acid (OA) (18:1n-9), respectively, which in turn can be esterified with G3P to form TG (Fig. 3). NADPH is a necessary co-factor during DNL and is produced by two cytoplasmic pathways. One pathway produces pyruvate and NADPH from the oxidative decarboxylation of malate by the malic enzyme. The other pathway, the pentose phosphate pathway, produces NADPH during: (i) the conversion of glucose-6-phosphate (G6P) to 6-phosphogluconate by the enzyme G6P dehydrogenase (G6PDH) and (ii) the synthesis of ribulose-5-phosphate from 6-phosphogluconate by the enzyme 6-phosphogluconate dehydrogenase (PGD) (Proença et al., 2014).

Many fish species experience both periods of food shortage and life stages with a high-energy demand (e.g. during reproduction or smoltification) requiring an increase in catabolic processes, which prevents further growth and mobilizes energy reserves such as those stored in glycogen, lipids and proteins (Martin et al., 2017; Navarro and Gutiérrez, 1995). In mammals, lipolysis or neutral hydrolysis of TG, requires three consecutive enzymatic steps. First, adipose triglyceride lipase (ATGL) catalyzes the initial stage of lipolysis, converting TG to one FA molecule and diacylglycerol (DG) (Lass et al., 2011). Then, hormone-sensitive lipase (HSL) is responsible for the hydrolysis of DG to another FA molecule and monoacylglycerol (MG) (Lass et al., 2011). Finally, monoacylglycerol lipase, hydrolyzes MG to the last FA molecule and glycerol (Lass et al., 2011) (Fig. 2).

Expansion of adipose tissue in fish

Hypertrophy

As mentioned before, AT grows by hypertrophy, hyperplasia or both. Adipocyte hypertrophy allows increased lipid load in existing adipocytes during periods of excess caloric intake, but that process is limited by physicochemical factors (Muir et al., 2016). In mammals, during rapid hypertrophic AT expansion, a hypoxic state results in the induction of hypoxia-inducible factor 1-alpha (HIF1α) which activates profibrotic genes. This gene activation results in the abnormal development of the extracellular matrix, provoking necrosis of the adipocytes and the subsequent attraction of M1 type macrophages, which ultimately lead to inflammation and metabolic dysfunction (Sun et al., 2013). Several studies in zebrafish have identified the potential mechanism(s) associated with hypertrophic growth in fish. Song and Cone developed a transgenic zebrafish that overexpresses the endogenous melanocortin antagonist agouti-related protein (AgRP), a well-known hypothalamic orexigenic peptide that increases food intake. This transgenic zebrafish exhibits obesity, increased linear growth, and visceral adipocyte hypertrophy, implicating the central nervous system (CNS) in regulating energy homeostasis (Song and Cone, 2007). Two studies, both using diet-induced obesity, revealed that some of the metabolic alterations identified in the mammalian systems are also conserved in zebrafish (Landgraf et al., 2017; Oka et al., 2010). Overfeeding zebrafish with 60 mg *Artemia* (150 calories), instead of a control 5 mg *Artemia* (20 calories) diet, for 8 weeks resulted in increased body mass index, hypertriglyceridemia and hepatosteatosis (Oka et al., 2010). In a

Journal of Experimental Biology (2018) 221, jeb161588. doi:10.1242/jeb.161588

recent study, Landgraf and collaborators also overfed zebrafish with either a normal-fat diet (NFD) of 60 mg *Artemia* or an isocaloric high-fat diet (HFD) for 8 weeks. They reported that both diets increased body weight and AT in comparison to control (diet of 5 mg *Artemia* fish). In addition, those fish that were fed the HFD also presented metabolic alterations (e.g. elevated blood glucose, plasma TG and cholesterol levels, ectopic accumulation of lipids in liver and muscle). These fish also had larger visceral adipocytes (adipocyte hypertrophy) but smaller subcutaneous adipocytes, indicating a metabolically unhealthy AT phenotype (Landgraf et al., 2017). In another study, using isolated adipocytes of the visceral AT from rainbow trout (*Oncorhynchus mykiss* Walbaum) fed with a high-energy diet to satiation for 8 weeks, the fish showed a certain degree of resistance to regulate leptin secretion when exposed to hormonal (insulin and ghrelin) or nutritional [leucine and eicosapentaenoic acid (EPA)] stimuli (Salmerón et al., 2015a). This is reminiscent of the altered leptin regulation seen in metabolic disorders described in rodent models fed high-fat diets (Buettner et al., 2007; Ceddia, 2005). In gilthead sea bream, fish were fed for 14 months with a fish oil diet or with an fish-oil-replacement diet containing 66% vegetable oil. Isolated adipocytes from the fish given the fish-oil-replacement diet showed increased lipolytic activity and adipocyte cell size (hypertrophy) and had both a reduced response to fatty acid treatments and lower insulin sensitivity (Cruz-Garcia et al., 2011). Therefore, overfed fish on a HFD or on a diet containing a high percentage of vegetable oils expand their AT mainly by adipocyte hypertrophy, and have metabolic disorders similar to those found in mammalian models when using similar experiments. These data also suggest that fish and mammals probably share a common molecular mechanism to induce adipocyte hypertrophy.

Hyperplasia

Adipocyte hyperplasia is based on the formation of new adipocytes from precursor cells and it is characterized by progenitor proliferation and differentiation (Hausman et al., 2001). Furthermore, it has been suggested that hyperplasia in fish occurs not only during early life stages (Umino et al., 1996) but also throughout life (Fauconneau et al., 1997; Spalding et al., 2008). In humans, hyperplasia occurs during all adult ages and at all levels of body mass index, with an approximately 10% of fat cells renewed annually (Spalding et al., 2008). Adipogenesis is characterized by the expression of a complex transcriptional network that results in the expression of several genes that are associated with the biochemical functions of a mature adipocyte, such as lipid uptake, transport and synthesis, hormonal sensitivity and the secretion of adipokines (Den Broeder et al., 2015; Rosen and MacDougald, 2006) (Fig. 4). Adipocyte differentiation is divided into two main phases, which requires the sequential induction of specific genes. First, the commitment of multipotent ASCs to the adipocyte lineage produces preadipocytes (the determination phase), which in turn will differentiate into mature adipocytes (the differentiation phase) (Fig. 4).

Determination phase

Peroxisome proliferator activated receptor gamma (PPARγ), a member of the superfamily of nuclear receptors, is considered the key regulator of adipogenesis, and it is necessary and sufficient to initiate and maintain this process (Rosen et al., 2000). PPARγ forms a heterodimer with the cis-retinoic acid receptor alpha (RXRα) and binds the promoters of several genes involved in FA and glucose metabolism (Lefterova et al., 2014). ASCs are maintained in an uncommitted state by two main inhibitory complexes: zinc-finger protein 521–early B-cell factor 1 (ZNF521–Ebf1) and wingless-

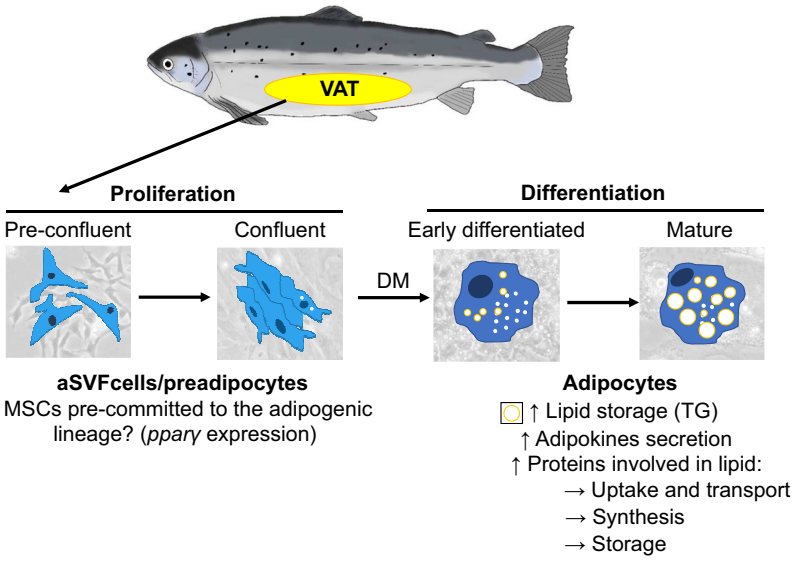

Fig. 4. Model of *in vitro* adipogenesis in fish using adipose stromal vascular fraction (aSVF) cells/preadipocyte primary cultures. Adipogenesis is based on the formation of new adipocytes from precursor cells and is characterized by progenitor proliferation and differentiation. Visceral adipose tissue (VAT) is formed by a heterogeneous population of cells named aSVF cells, which includes multipotent mesenchymal stem cells (MSCs), also known as adipose tissue-derived stem cells (ASCs) or preadipocytes, that have the ability to differentiate into adipocytes. Fish aSVF cells/preadipocytes are isolated from VAT, disaggregated by mechanical and enzymatic methods, and grown under controlled conditions outside the body. These isolated cells seem to be pre-committed to the adipogenic lineage because they express the adipogenic *peroxisome proliferator activated receptor gamma* (*pparγ*) gene without being subjected to adipogenic differentiation media. The addition of a differentiation medium to the preadipocytes in culture induces the expression adipogenic transcription factors, which initiate the adipocyte gene program. Adipocyte differentiation is sustained using an adipogenic medium (AM) and it is characterized by an increase in the lipid accumulation in form of triglycerides (TGs), by the secretion of adipokines and by the expression of proteins involved in lipid uptake, transport, synthesis and storage.

type MMTV integration site (WNT) family member 2 (WISP2)–ZNF423 (Gustafson et al., 2015). The disassociation of these complexes allows ZNF423 and Ebf1/2 to enter the nucleus and activate *pparγ* transcription, producing committed preadipocytes (Gustafson et al., 2015). The bone morphogenetic protein 4 (BMP4), which is secreted by differentiated (pre)adipocytes, induces the adipogenic commitment of ASCs in a paracrine fashion, activating its receptor which leads to the dissociation of the WISP2–ZNF423 complex (Gustafson et al., 2015). Two of these important transcription factors, ZNF521 and ZNF423, are present in the zebrafish genome (Gene IDs: 799182 and 325643, respectively) and *znf423* can already be detected 22 h post fertilization in the forebrain, hindbrain, eye and spinal cord of zebrafish embryos using *in situ* hybridization (Ariza-Cosano et al., 2012). In another study, using Atlantic salmon primary aSVF cells in culture, the expression of *bmp4* increased after the induction of adipocyte differentiation, suggesting that a similar transcriptional regulation might occur in fish (Todorčević et al., 2010). Nevertheless, the exact mechanism(s) of ASC commitment in fish remain to be elucidated. Visceral AT is usually the largest fat depot in the fish body and is the tissue targeted in order to isolate aSVF cells for these primary cultures. Interestingly, the aSVF cells isolated for these primary cultures seem to be pre-committed to the adipogenic lineage because they express the adipogenic *pparγ* gene before differentiation (Bouraoui et al., 2008; Salmerón et al., 2016; Todorčević et al., 2010) (Fig. 4) and they accumulate small LD in their cytoplasm even without being subjected to adipogenic differentiation medium (Salmerón et al., 2013; Vegusdal et al., 2003; Ytteborg et al., 2015).

Differentiation phase

Studies of mammalian cell lines (e.g. murine 3T3-L1 preadipocyte cell lines) have revealed that at least two waves of activation of transcription factors are necessary to complete the adipogenic process. First, the addition of a differentiation cocktail to the preadipocytes in culture activates the transcription factor cyclic AMP (cAMP) response element binding protein (CREB) and induces the expression of CCAAT-enhancer-binding proteins (c/ebp) β and δ, of the glucocorticoid receptor, and of Signal transducer and activator of transcription 5A (STAT5A) (Lefterova et al., 2014). CREB activation, in turn, induces the expression of other transcription factor genes, including *pparγ* and *c/ebpα*, which initiate the adipocyte gene program (Lefterova et al., 2014). Both PPARγ and C/EBPα coordinate the expression of adipocyte-specific genes [e.g. *fabp4*, *adiponectin*, the Glucose transporter 4 gene (*glut4*) and *lpl*], some of which are involved in the later stages of adipocyte differentiation (Gustafson et al., 2015). When working with *in vitro* models of preadipocytes, it is necessary to add to the medium some of the compounds that activate the genes involved in adipogenesis. Generally, three components are used in the cocktail for differentiation in mammalian cells: insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX) (Scott et al., 2011).

In general, fish primary aSVF cell cultures also require the addition of a differentiation medium (DM) to induce adipogenesis (Fig. 4), but there are substantial differences in the growth medium (GM) or the DM formulation depending on the species. Some fish primary preadipocyte cell cultures use GM with L-15 Medium (Leibovitz) [Atlantic salmon (Todorčević et al., 2010), rainbow trout (Salmerón et al., 2015b), and common carp (*Cyprinus carpio* L.) (Ljubojevic et al., 2014)], with Dulbecco's Modified Eagle's Medium (DMEM) [gilthead sea bream (*Sparus aurata* L.) (Salmerón et al., 2013)] or with DMEM/Ham's Nutrient Mixture F-12 (DMEM/F12) [red sea bream: *Pagrus major* (Temminck & Schlegel), (Oku and Umino, 2008); large yellow croaker: *Pseudosciaena crocea* (Richardson), (Wang et al., 2012); and grass carp: *Ctenopharyngodon idella* (Valenciennes), (Li, 2012)]. In general, GM contain 10% fetal bovine serum, supplemented for some species with HEPES, L-glutamine, antibiotic or antimycotic solution and/or NaCl (Ljubojevic et al., 2014; Oku and Umino, 2008; Salmerón et al., 2013, 2015b; Todorčević et al., 2010; Wang et al., 2012). Adipocyte induction in these fish preadipocytes cultures is generally by means of a DM-containing GM with species-specific supplementation. Common to all fish DM is the inclusion of a lipid source, such as a lipid mixture or linoleic acid (which is included in DMEM/F12 medium). Although unnecessary in mammalian preadipocyte cultures, this lipid source is necessary in fish preadipocyte cultures to induce adipocyte differentiation fully, probably acting as an adipogenic factor (e.g. providing ligands that activate PPARγ) and providing cholesterol and lipids for LD development (Bouraoui et al., 2008; Salmerón et al., 2013; Vegusdal et al., 2003; Wang et al., 2012). Other supplements (e.g. hormones) improve the differentiation capacity of the medium (Oku et al., 2006; Todorčević et al., 2008). The biological characteristics of each fish species determine the optimal cell culture conditions, from incubation temperature (which will differ for cold-water and warm-water species) to GM and DM compositions (which will differ for freshwater or marine species). These species-specific culture requirements complicate the creation of a standardized protocol that would facilitate the comparison and interpretation of data derived from preadipocyte cultures in fish.

Molecular profiling during adipocyte differentiation in fish

Four clear stages can be indentified in primary cultures of fish preadipocytes: pre-confluent preadipocytes (usually from day 1 to 7), confluent preadipocytes (after approximately 1 week in culture), early differentiated adipocytes (in the first days after induction of differentiation) and mature adipocytes (around 1 week after induction of differentiation). After 1 day in culture, attached cells generally present a small spindle- and star-shaped morphology similar to that of fibroblasts (Salmerón et al., 2013; Vegusdal et al., 2003) (Fig. 4) and comprise a Proliferating Cell Nuclear Antigen (PCNA)-negative population (Vegusdal et al., 2003) with raised expression of non-adipogenic cell markers (e.g. markers for MSCs, vasculature and immune cells) (Todorčević et al., 2010) (Table 1). Soon afterwards, cells begin to proliferate, and at day 4, they are PCNA-positive with more elongated cytoplasm and developing connections with neighboring cells (Vegusdal et al., 2003). Surprisingly, these cells already express some adipocyte-related genes (e.g. *pparγ* and *c/ebpβ*) (Todorčević et al., 2010) (Table 1). Confluent preadipocytes are also proliferative and show an extensive and relatively homogeneous cytoplasm, devoid of LDs (with some exceptions as mentioned before) (Salmerón et al., 2013; Vegusdal et al., 2003; Wang et al., 2012). These confluent cells are characterized by: (i) the downregulation of *hsl* and *pparα* gene expression in gilthead sea bream (Salmerón et al., 2016) and markers of non-adipogenic cells in Atlantic salmon (Todorčević et al., 2010), and (ii) upregulation of *transgelin*, *fas* and *g6pdh* in Atlantic salmon and in gilthead sea bream (Salmerón et al., 2016; Todorčević et al., 2010) (Table 1). The induction of differentiation, by means of a DM, produces morphological and biochemical changes in the preadipocytes which become more rounded and filled with LD. Atlantic salmon cells respond quickly to the induction, accumulating many small LDs in the cytoplasm by as soon as 24 h after induction (Vegusdal et al., 2003), and after 3–4 days in

Table 1. Molecular profile during *in vitro* adipogenesis in fish using adipose stromal vascular fraction (aSVF) cells/preadipocyte primary cultures

Organism	Pre-confluent preadipocytes	Confluent preadipocytes	Early differentiated adipocytes	Mature adipocytes
Atlantic salmon (*Salmo salar*)	↑ Markers of vascular cells, macrophages, lymphocytes, and preadipocytes, *pparγ* and *c/ebpβ*[1]	↑ Adipogenic markers and ↓ markers of other cell lineages[1] ↑ *transgelin* and *fas*[1]	↓ *tnfα*, *myoD*[1] ↑ *c/ebpα*[1,2], *pparγ short*[3], *cpt-II*[3], *acd*[1,3], *fatp1*[2,3], *fabp3*[3], *sr-bI*[3], *bmp4*[1] PPARγ, C/EBPα and leptin presence[4] ↑GPDH activity[4]	↑ *c/ebpδ*[1,2], *lpl*[1,3], *fas*[1], *fabp11*[2], *mtp*[1,3], *g6pdh*, *pfk*, *pk*, *mdh*, *pgd*, *pepck-c*, adipokines and various stress response-related genes[1]
Rainbow trout (*Oncorhynchus mykiss*)			↓ *adiponectin*[5] ↑ *lpl*[6,7], *fatp1*[7], *adipoR1*[5], *adipoR2*[5], *lxr*[8], PPARγ[9], IGF-IR[10] and leptin[7] C/EBPα[9], ghrelin[7] and GHS-R1a presence[7] ↑ GPDH activity[9]	
Red sea bream (*Pagrus major*)			↑ *lpl*[11], *lpl2*[12] and *pparα*[12] ↓*glut1* and *βactin*[12]	↑ *fas*, *d6des*, *scda* and *scdb*[12]
Gilthead sea bream (*Sparus aurata*)		↓ *hsl* and *pparα*[13] ↑ *g6pdh*[13]	↓ *lpl*, *gapdh2* and *pparγ*[13]	↑ *g6pdh* ↓ *gapdh1* and *pparβ*[13] GAPDH presence[13]
Large yellow croaker (*Pseudosciaena crocea*)			↑ *lpl* and *pparγ*[14] ↑ GPDH activity[14]	
Grass carp (*Ctenopharyngodon idella*)				↑ *pparγ*[15] ↑ GPDH activity[15]

↑, increase; ↓, decrease. Pre-confluent preadipocytes are cells in the first stage of culture before cell confluence. Confluent preadipocytes are cells in the second stage of culture before adipogenesis induction, with partial or complete confluence. Early differentiated adipocytes are cells in the third stage of culture, after the addition of an adipogenic differentiation medium, with cells presenting small lipid droplets (LD). Mature adipocytes are cells in the last stage of culture and are round-shaped cells with large LD.

Abbreviations: *acd*, acyl-CoA dehydrogenase gene; *adipoR1* and *2*, adiponectin receptor 1 and 2 genes; *bmp4*, bone morphogenetic protein 4 gene; *c/ebpα*, *β* and *δ*, CCAAT/enhancer-binding protein alpha, beta and delta genes; *cpt-II*, carnitine palmitoyl transferase II gene; *d6des*, delta-6-desaturase gene; *fas*, fatty acid synthase gene; *fabp3*, fatty acid binding protein 3 gene; *fatp1* and *11*, fatty acid transporter protein 1 and 11 genes; *g6pdh*, glucose-6-phosphate dehydrogenase gene; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; *gapdh1* and *2*, glyceraldehyde 3-phosphate dehydrogenase 1 and 2 genes; GHS-R1a, growth hormone secretagogue receptor 1a; *glut1*, glucose transporter 1 gene; GPDH, glycerol-3-phosphate dehydrogenase; *hsl*, hormone-sensitive lipase gene; IGF-IR, insulin-like growth factor I receptor; *lpl*, lipoprotein lipase gene; *lxr*, liver X receptor gene; *mdh*, malate dehydrogenase gene; *mtp*, microsomal triglyceride transfer protein gene; *myoD*, myogenic factor D gene; *pepck-c*, cytoplasmic phosphoenolpyruvate carboxykinase gene; *pfk*, phosphofructokinase gene; *pgd*, 6-phosphogluconate dehydrogenase gene; *pk*, pyruvate kinase gene; *pparα*, β and γ, peroxisome proliferator activated receptor alpha, beta and gamma genes; *scda* and *b*, stearoylCoA desaturase a and b genes; *sr-bI*, scavenger receptor class B type I gene; *tnfα*, tumor necrosis factor α gene.

Atlantic salmon: (1) Todorčević et al., 2010; (2) Huang et al., 2010; (3) Todorčević et al., 2008; (4) Vegusdal et al., 2003. Rainbow trout: (5) Sánchez-Gurmaches et al., 2012; (6) Bouraoui et al., 2012; (7) Salmerón et al., 2015b; (8) Cruz-Garcia et al., 2012; (9) Bouraoui et al., 2008; (10) Bouraoui et al., 2010. Red Sea bream: (11) Oku et al., 2006; (12) Oku and Umino, 2008. Gilthead sea bream: (13) Salmerón et al., 2016. Large yellow croaker: (14) Wang et al., 2012. Grass carp: (15) Li, 2012.

DM, almost all of the cells of the different fish species contain many large LDs of different sizes (Salmerón et al., 2013; Vegusdal et al., 2003; Wang et al., 2012). After 1 week post induction, the number of round-shaped cells with larger LD increases, probably due to the fusion of small LD. These cells are considered mature adipocytes. In Atlantic salmon, these mature cells have a reduced number of mitochondria and an eccentric nucleus, and they are easily detached from the culture plate so that they float in the medium (Todorčević et al., 2010; Vegusdal et al., 2003). Similar morphological observations have been reported *in vivo* in zebrafish visceral adipocytes (Flynn et al., 2009), suggesting that these *in vitro* fish models represent what occurs at the morphological level *in vivo*.

Analysis of GPDH activity and of the expression of specific adipocyte-related genes or their protein products are some of the most common markers used to evaluate adipocyte differentiation. GPDH is a key enzyme in the synthesis of TG. In fish, preadipocytes that are exposed to DM increase GPDH activity through differentiation (Li, 2012), showing greater activity in cells growing in DM than in those cultivated only in GM (Bouraoui et al., 2008; Vegusdal et al., 2003; Wang et al., 2012) (Table 1), with lipids stimulating (Bouraoui et al., 2008; Wang et al., 2012) and TNFα inhibiting GPDH activity (Bouraoui et al., 2008) (Table 2). These studies demonstrate that adipocyte differentiation in fish requires an enhanced level of lipid synthesis.

Salmoniformes

Early differentiated adipocytes from Atlantic salmon are characterized by a downregulation of the transcription factors TNFα (a well know adipogenesis inhibitor) and myogenic factor D (myoD) (a marker of myogenic commitment) (Todorčević et al., 2010) (Table 1). Furthermore, these early differentiated cells present an upregulation of genes involved in adipogenesis, such as the adipogenic transcription factor genes *c/ebpα* (Huang et al., 2010; Todorčević et al., 2010) and *pparγ short* (a *pparγ* splice variant that lacks the first 102 nucleotides of exon 3 of *pparγ long*) (Todorčević et al., 2008), mitochondrial β-oxidation pathway genes [encoding acyl-CoA dehydrogenase (*acd*) (Todorčević et al., 2008, 2010) and carnitine palmitoyl transferase II (*cpt-II*) (Todorčević et al., 2008)], lipid metabolism-related genes [*fatp1* (Huang et al., 2010; Todorčević et al., 2008), *fabp3* and *sr-bI* (which encodes *scavenger receptor class B type I*) (Todorčević et al., 2008)], and other genes such as *bmp4* (Todorčević et al., 2010) (Table 1). Similar results have also been reported at the protein level, where early differentiated adipocytes express the transcription factors PPARγ and C/EBPα in the nuclei and the adipokine leptin in the

Table 2. Effects of adipokines on fish adipogenesis

Organism	Treatment	Proliferation	Differentiation
Rainbow trout (*Oncorhynchus mykiss*)	TNFα	10–100 ng ml^{-1}: no effect[1]	100 ng ml^{-1}: ↓GPDH activity[1] and PPARγ protein expression[2] 100 ng ml^{-1}: ↑*adipoR1* mRNA and glucose uptake[2] 100 ng ml^{-1}+1 µmol l^{-1} insulin: ↑glucose uptake[2] 100 ng ml^{-1}+100 nmol l^{-1} IGF-I: ↑glucose uptake[2]
	Adiponectin	ND	2.5 µg ml^{-1}: ↑glucose uptake[2] 2.5 µg ml^{-1}+1 µmol l^{-1} insulin: ↑*pparγ* mRNA[2] 2.5 µg ml^{-1}+100 nmol l^{-1} IGF-I: ↑glucose uptake[2]
	Leptin	100 nmol l^{-1}: no effect[3]	100 nmol l^{-1}: ↓*lpl* mRNA[3]
	Ghrelin	10 nmol l^{-1}: no effect[3]	10 nmol l^{-1}: no effect[3]
Large yellow croaker (*Pseudosciaena crocea*)	TNFα	100 ng ml^{-1}: suppressed preadipocyte proliferation	1–100 ng ml^{-1}: ↑glycerol release during differentiation and lipolysis[4] 1–100 ng ml^{-1}: ↓*atgl*, *pparα* and *pparγ* mRNA; 1 ng ml^{-1}: ↑*fas*, ↓*lpl* mRNA during differentiation[4] 1–100 ng ml^{-1}: ↓*atgl* mRNA; 1 ng ml^{-1}: ↑*pparα* and *pparγ* mRNA; 10 ng ml^{-1}: ↑*fas* mRNA; 100 ng ml^{-1}: ↓*lpl* mRNA during lipolysis[4]

↑, increase; ↓, decrease; ND, not determined. Adipose stromal vascular fraction (aSVF) cells/preadipocyte primary cultures. Proliferation: proliferative cells in culture before and during confluence. Differentiation: cells after adipogenesis induction with cells containing small or large lipid droplets.
Abbreviations: *adipoR1*, adiponectin receptor 1 gene; *atgl*, adipose triglyceride lipase gene; *fas*, fatty acid synthase gene; GPDH, glycerol-3-phosphate dehydrogenase; IGF-I, insulin-like growth factor I; *lpl*, lipoprotein lipase gene; PPARα and γ, peroxisome proliferator activated receptor alpha and gamma; TNFα, tumor necrosis factor α.
Rainbow trout: (1) Bouraoui et al., 2008; (2) Bou et al., 2014; (3) Salmerón et al., 2015b. Large yellow croaker: (4) Wang et al., 2012.

cytoplasm, particularly in a concentrated perinuclear region of the cytoplasm (Vegusdal et al., 2003) (Table 1). During the later phase of differentiation, Atlantic salmon adipocytes present an upregulation in the expression of genes involved in terminal differentiation, such as adipogenic transcription factor genes [*c/epbδ* (Huang et al., 2010; Todorčević et al., 2010)], lipid and glucose metabolism-related genes [*lpl* (Todorčević et al., 2008, 2010), *fas* (Todorčević et al., 2010), *fabp11* (Huang et al., 2010), *mtp* (encoding microsomal triglyceride transfer protein) (Todorčević et al., 2008, 2010), *g6pdh*, *phosphofructokinase* (*pfk*), *pyruvate kinase* (*pk*), *mdh*, *pgd* and *pepck-c* (Todorčević et al., 2010)], adipokine genes (*adipsin* and *visfatin*) and various stress-response-related genes (Todorčević et al., 2010) (Table 1). It is important to notice that in the mature adipocytes of Atlantic salmon, the expression of genes requiring NADPH, a reducing agent, such as those involved in cellular antioxidant defense [e.g. the glutathione reductase gene (*gsr*)] or fatty acid synthesis (e.g. *fas*), is upregulated during differentiation in parallel with the expression of genes encoding NADPH-producing enzymes (e.g. *g6pdh* and *pgd*) (Todorčević et al., 2010) (Table 1).

Differentiated adipocytes from rainbow trout are characterized by downregulated expression of the adipokine gene *adiponectin* (Sánchez-Gurmaches et al., 2012) and upregulation of genes encoding lipid-metabolism-related enzymes [*lpl* (Bouraoui et al., 2012; Salmerón et al., 2015b) and *fatp1* (Salmerón et al., 2015b)], both adiponectin receptors (*adipoR1* and *adipoR2*) (Sánchez-Gurmaches et al., 2012) and the transcription factor liver X receptor (*lxr*) (Cruz-Garcia et al., 2012) (Table 1). At the protein level, these differentiated adipocytes also express ghrelin and its receptor, the growth hormone secretagogue receptor 1a (GHS-R1a) (Table 1). Furthermore, these differentiated adipocytes secrete more leptin (Salmerón et al., 2015b) and express more PPARγ, C/EBPα (Bouraoui et al., 2008) and insulin-like growth factor I receptor (IGF-IR) (Bouraoui et al., 2010) protein than do non-differentiated preadipocytes (Table 1).

Perciformes

Early differentiated adipocytes from Red Sea bream are transcriptionally characterized by the downregulation of *glut1* and *βactin* (Oku and Umino, 2008) and by upregulation of the transcription factor gene *pparα* (Oku and Umino, 2008) and lipogenic genes (*lpl1* and *lpl2*) (Oku et al., 2006; Oku and Umino, 2008) (Table 1). Expression of the lipogenic genes *fas*, *d6des* (encoding delta-6-desaturase), *scda* and *scdb* (encoding stearoylCoA desaturases) is upregulated in Red Sea bream mature adipocytes, suggesting an increase in lipid synthesis (Oku and Umino, 2008) (Table 1). Large yellow croaker preadipocytes in culture already show increased expression of the adipocyte marker genes *lpl* and *pparγ* 3 days after the induction of differentiation (Wang et al., 2012) (Table 1). In grass carp, *pparγ* gene expression is also increased during the last stage of adipocyte differentiation (Li, 2012) (Table 1). Curiously, the genetic profile of gilthead sea bream during adipogenesis is characterized by the downregulation of well-known adipogenic gene markers (e.g. *lpl*, *gapdh1*, *gapdh2*, *pparγ*, and *pparβ*) and the increase of *g6pdh*, suggesting some kind of species-specific regulation of gene expression (Salmerón et al., 2016) (Table 1). Altogether, these data suggest that, in general, the expression of *pparγ*, *lpl* and *fatp1* genes and the presence of PPARγ and C/EBPα proteins are suitable molecular markers for the evaluation of adipogenesis in fish.

Effects of adipokines, growth factors, fatty acids and other factors on fish adipogenesis

Adipokines (TNFα, adiponectin, leptin and ghrelin)

AT is characterized by the production of adipokines, which can act in an endocrine, paracrine and autocrine fashion. The effects of adipokines in fish adipogenesis have been investigated in aSVF primary cultures of rainbow trout, with studies examining the effects of recombinant human TNFα (Bou et al., 2014; Bouraoui et al., 2008), human globular adiponectin (Bou et al., 2014) and recombinant rainbow trout leptin and ghrelin (Salmerón et al., 2015b). The effects of human TNFα have also been studied in large yellow croaker (Wang et al., 2012) (Table 2). TNFα does not affect preadipocyte proliferation in rainbow trout (Bouraoui et al., 2008) but suppresses preadipocyte proliferation at higher doses (100 ng ml^{-1}) in large yellow croaker (Wang et al., 2012) (Table 2). In differentiated adipocytes in culture, TNFα at a higher dose (100 ng ml^{-1}) inhibits adipogenesis by decreasing the activity of GPDH [in rainbow trout (Bouraoui et al., 2008) and in large yellow croaker (Wang et al., 2012)], by decreasing the PPARγ

Table 3. Effects of growth factors on fish adipogenesis

Organism	Treatment	Proliferation	Differentiation
Rainbow trout (*Oncorhynchus mykiss*)	Insulin	1000 nmol l^{-1}: ↑proliferation[1]	1 µmol l^{-1}: ↑*adiponectin* mRNA and glucose uptake and ↓*adipoR1* mRNA[2] 10 nmol l^{-1} and 1.7 µmol l^{-1}: ↑*lpl* mRNA[3] 1.7 µmol l^{-1} insulin+1 or 5 µmol l^{-1} troglitazone: ↑*lpl* mRNA[3] 1 µmol l^{-1} insulin+1 µmol l^{-1} troglitazone: ↑lipid accumulation[3]
	IGF-I	100 nmol l^{-1}: ↑proliferation[4]	100 nmol l^{-1}: ↑glucose uptake[2,5]
Large yellow croaker (*Pseudosciaena crocea*)	Insulin	0.5–50 µg ml^{-1}: ↑proliferation[6]	0.5–50 µg ml^{-1}: ↑GPDH activity ↓lipolysis[6] 0.5 µg ml^{-1}: ↑*lpl* mRNA; 5 µg ml^{-1}: ↓*atgl* and *pparα*, ↑*fas* mRNA during differentiation[6] 0.5–50 µg ml^{-1}: ↓*atgl* mRNA; 0.5 µg ml^{-1}: ↓*lpl* mRNA; 5–50 µg ml^{-1}: ↑*lpl* and *pparγ* mRNA; 50 µg ml^{-1}: ↑*pparα* mRNA during lipolysis[6]
Red sea bream (*Pagrus major*)	Insulin	ND	5 µg ml^{-1}: ↑lipid accumulation[7] 50 µg ml^{-1}: ↑*lpl* mRNA[7] 5 µg ml^{-1}+20 nmol l^{-1} triiodothyronine (T3): ↑*lpl* mRNA[7]
Gilthead sea bream (*Sparus aurata*)	Insulin	10–1000 nmol l^{-1}: ↑proliferation[8]	1000 nmol l^{-1}: no effect[8]
	IGF-I	10 and 100 nmol l^{-1}: ↑proliferation[8]	100 nmol l^{-1}: ↑lipid content[8]
	GH	1 and 10 nmol l^{-1}: ↑proliferation[8]	ND

↑, increase; ↓, decrease; ND, not determined. Adipose stromal vascular fraction (aSVF) cells/preadipocyte primary cultures. Proliferation: proliferative cells in culture before and during confluence. Differentiation: cells after adipogenesis induction containing small or large lipid droplets.
Abbreviations: *adipoR1*, adiponectin receptor 1 gene; *atgl*, adipose triglyceride lipase gene; *fas*, fatty acid synthase gene; GPDH, glycerol-3-phosphate dehydrogenase; IGF-I, insulin-like growth factor I; *lpl*, lipoprotein lipase gene; *pparα* and *γ*, peroxisome proliferator activated receptor alpha and gamma genes.
Rainbow trout: (1) Salmerón et al., 2015a,b; (2) Bou et al., 2014; (3) Bouraoui et al., 2012; (4) Bouraoui et al., 2008; (5) Bouraoui et al., 2010. Large yellow croaker: (6) Wang et al., 2012. Red sea bream: (7) Oku et al., 2006. Gilthead sea bream: (8) Salmerón et al., 2013.

protein level [in rainbow trout (Bou et al., 2014)] and by downregulating the expression of *atgl*, *pparα* and *pparγ* genes during adipocyte differentiation [using 1 to 100 ng ml^{-1} in large yellow croaker (Wang et al., 2012)] (Table 2). Therefore, TNFα seems to be an anti-adipogenic adipokine in fish as it is in mammals. The effect of adiponectin on fish adipogenesis has not yet been explored. In rainbow trout preadipocyte cultures, insulin (at 1 µmol l^{-1}) has different effects. Alone, it upregulates adiponectin gene expression while downregulating the adiponectin receptor (AdipoR1) (Bou et al., 2014) (Table 3). In combination with adiponectin (at 2.5 µg ml^{-1}), insulin also upregulates *pparγ* expression (Bou et al., 2014) (Table 2). By contrast, TNFα (100 ng ml^{-1}) upregulates *adipoR1* expression (Bou et al., 2014) (Table 2). Neither leptin nor ghrelin hormone affects preadipocyte proliferation or differentiation in rainbow trout; however, leptin decreases the expression of *lpl*, suggesting that leptin might have an inhibitory role during adipocyte differentiation (Salmerón et al., 2015b) (Table 2).

Growth factors (insulin, IGF-I and GH)

The effects of the growth factors insulin, insulin-like growth factor I (IGF-I) and growth hormone (GH) in fish adipogenesis have been explored using primary preadipocyte cultures of Red Sea bream (Oku et al., 2006), rainbow trout (Bouraoui et al., 2008, 2010, 2012), large yellow croaker (Wang et al., 2012) and gilthead sea bream (Salmerón et al., 2013) (Table 3). In Red Sea bream, bovine insulin (at 5 µg ml^{-1}) increases both the accumulation of lipid (TG) and *lpl* gene expression (Oku et al., 2006) (Table 3). In rainbow trout, porcine insulin (at 1000 nmol l^{-1}) (Salmerón et al., 2015b) and recombinant human IGF-I (at 100 nmol l^{-1}) (Bouraoui et al., 2008) have been shown to promote preadipocyte proliferation [evidence suggests that MAPK activation mediates this response (Bouraoui et al., 2010)], and to increase adipogenesis through the induction of *lpl* expression (Bouraoui et al., 2012) (Table 3). In large yellow croaker, insulin increases the proliferation of preadipocytes (Table 3). It also has an adipogenic effects by stimulating GPDH activity and by upregulating the expression of adipogenic genes (e.g. *lpl* and *fas*) while downregulating the lipolytic enzyme *atgl* (Wang et al., 2012) (Table 3). Finally, in gilthead sea bream, recombinant human GH, recombinant human IGF-I and porcine insulin stimulates proliferation (Salmerón et al., 2013) (Table 3). In addition, IGF-I also increased the lipid content (Salmerón et al., 2013) (Table 3). In conclusion, GH, insulin and IGF-I stimulate proliferation, whereas only insulin and IGF-I promote adipogenesis, in fish.

Fatty acids (OA, EPA, DHA and PA)

Almost half (44.1%) of the seafood produced worldwide today comes from aquaculture farms (SOFIA 2016), and experts predict that this proportion will exceed 65% by 2030 (APROMAR-ESACUA, 2013). Global food production will need to grow by 70% between 2010 and 2050 in order to provide enough food for an expected global population of 9.1 billion people (FAO, 2009). Mariculture, or the culture of marine organisms, is now expected contribute to the future demand for food, but its success requires improvement of the health of the stocks and their supporting marine ecosystems as well as changes to feed sources, so that human foods are not used and currently untapped feeds (e.g. microbial or planktonic products) are exploited (Watson et al., 2015). Lipids are excellent sources of metabolic energy and supply essential FAs that can support an optimal growth rate in fish (Tocher, 2003). Lipids, especially those from plants, are frequently used in aquacultural fish diets, partially to spare the unsustainable and relatively expensive use of fish meal and fish oil for conversion into muscle or flesh (Sprague et al., 2016). The use of hyperlipidic diets and the low swimming activity have, however, caused an increases in the total body lipid content and the mass of AT in cultured fish, with negative impacts for aquaculture in terms of production levels, consumer appreciation, half-life of the product and animal welfare (He et al., 2015; Meguro et al., 2015; Salmerón et al., 2015a). Currently, feed for carnivorous fish contains more lipids from plant sources [which are relatively rich in n-6 and n-9 polyunsaturated fatty acids (PUFAs) such as OA] than lipids of marine origin [which have higher levels of n-3 long-chain (LC)-PUFAs such as EPA or docosahexaenoic acid (DHA)], resulting in changes to the fatty acid composition of the farmed fish (Sprague et al., 2016).

The effects of OA, EPA, DHA and PA in fish adipogenesis have been explored in aSVF primary cultures of Atlantic salmon (Bou et al., 2016; Huang et al., 2010; Todorčević et al., 2008) and of large yellow croaker (Wang et al., 2012). In Atlantic salmon, mature adipocytes treated with OA accumulate more lipids than those incubated with the same dose of EPA or DHA (all FAs at 600 µmol l^{-1}) (Todorčević et al., 2008). The same difference was also detected in early, middle and mature adipocytes in experiments using radiolabeled OA, EPA and DHA substrates, suggesting that OA leads to higher lipid deposition (mainly in the form of TG) and adipogenesis than does EPA or DHA (Todorčević et al., 2008). In a follow-up study in Atlantic salmon, early differentiated adipocytes treated with EPA and/or DHA had downregulated expression of pro-adipogenic genes (e.g. *c/ebpβ*, *c/ebpδ*, *pparβ* and *fatp1*) in contrast to those treated with OA (all FAs at 600 µmol l^{-1}), suggesting that EPA and DHA but not OA have an anti-adipogenic role in Atlantic salmon. In a recent study, Bou and collaborators demonstrated that mature adipocytes treated with PA (at 40 or 80 µmol l^{-1}) accumulate more intracellular lipids (mainly in form of TG) than those treated with glucose (1 or 5.5 mmol l^{-1}) (Bou et al., 2016). In the same study, the expression of *creb*, *cpt1*, *acyl-CoA oxidase* (*aco*), *acc* and *transketolase* (*tkt*) genes in mature adipocytes was downregulated with PA, while *pgd* and *pepck-c* gene expression was upregulated (Bou et al., 2016), suggesting that PA activates the adipogenic pathway in Atlantic salmon adipocytes. In large yellow croaker DHA acts as an anti-adipogenic factor that inhibits GPDH activity and downregulates the expression of adipogenic genes such as *lpl*, *fas*, *pparα* and *pparγ* (using DHA at 50 µmol l^{-1}), while increasing the expression of lipolytic genes such as *atgl* (using DHA at 50 and 200 µmol l^{-1}) (Wang et al., 2012). In conclusion, EPA and DHA are anti-adipogenic and lipolytic factors in fish adipogenesis, whereas OA promotes lipid accumulation and adipogenesis.

PPAR agonists

The effects of different PPAR agonists in fish adipogenesis have been explored in Red Sea bream (Oku et al., 2009; Oku and Umino, 2008) and in rainbow trout (Bouraoui et al., 2012) preadipocyte primary cultures. In Red Sea bream, the PPAR agonist 2-bromopalmitate upregulates the expression of the *pparγ* gene and of related adipogenic genes (e.g. *lpl1*, *fas*, *scda* and *scdb*) (Oku and Umino, 2008). In rainbow trout, troglitazone (another PPAR agonist) upregulates expression of the adipogenic gene *lpl* and, in combination with insulin, increases lipid accumulation (Bouraoui et al., 2012). Therefore, these two PPAR agonists seem to be good candidates to induce adipogenesis in fish, although more studies are necessary to elucidate their exact mechanism of action.

Conclusions

Over the past decade, different biological tools have been developed to study adipogenesis in fish: from *in vitro* primary cultures of aSVF to an *in vivo* animal model (zebrafish). Teleost fish possess AT that has molecular, morphological and functional characteristics that are similar to those of other vertebrates. This AT contains different cell types among which the adipocytes, or the lipid-accumulating cells, are the most abundant and well-characterized. These adipocytes appear anatomically several days post-fertilization in zebrafish, and they can be differentiated *in vitro* from different precursor cells of cultured fish species. Fish adipocytes express the transcription factors that are required for the adipogenic process, as well as the enzymes involved in lipid metabolism. Interestingly, the regulation of adipogenesis is well conserved across species, having similarities in fish and mammals (e.g. insulin promotes and TNFα inhibits adipogenesis in both). It is important to notice that marine PUFAs (e.g. EPA and DHA) have an antiadipogenic effect on adipocyte development in fish; however, current diets contain higher levels of vegetable oils (e.g. OA) which stimulate adipogenesis in fish. The present Review provides main insights about recent discoveries in physiology and biochemistry of AT in aquacultured fish, highlighting the potential regulators of its development. These discoveries not only are helpful to better understand adipogenesis in fish, but also may contribute to identifying potential anti-obesogenic compounds to incorporate into fish diets in order to reduce the detrimental effects of excessive fat in fish.

Competing interests

The author declares no competing or financial interests.

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