Studies on the function and regulation of glucose transporters GLUT2 and GLUT4 in teleost fish

Estudios sobre la función y regulación de los transportadores de glucosa GLUT2 y GLUT4 en peces teleósteos

Rubén Marín Juez

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UNIVERSIDAD DE BARCELONA
FACULTAD DE BIOLOGÍA
DEPARTAMENTO DE FISIOLOGÍA E INMUNOLOGÍA

Studies on the function and regulation of glucose transporters GLUT2 and GLUT4 in teleost fish

Estudios sobre la función y regulación de los transportadores de glucosa GLUT2 y GLUT4 en peces teleósteos

Memoria presentada por
Rubén Marín Juez
Para optar al grado de
Doctor por la Universidad de Barcelona

Tesis realizada bajo la dirección del Dr. Josep Planas Vilarnau del Departamento de Fisiología e Inmunología, Facultad de Biología

Adscrita al Departamento de Fisiología e Inmunología, Facultad de Biología, Universidad de Barcelona, programa de Acuicultura

Dr Josep Planas Vilarnau **Rubén Marín Juez**

Barcelona , 6 de Julio 2012

“Science is the poetry of reality”
Richard Dawkins

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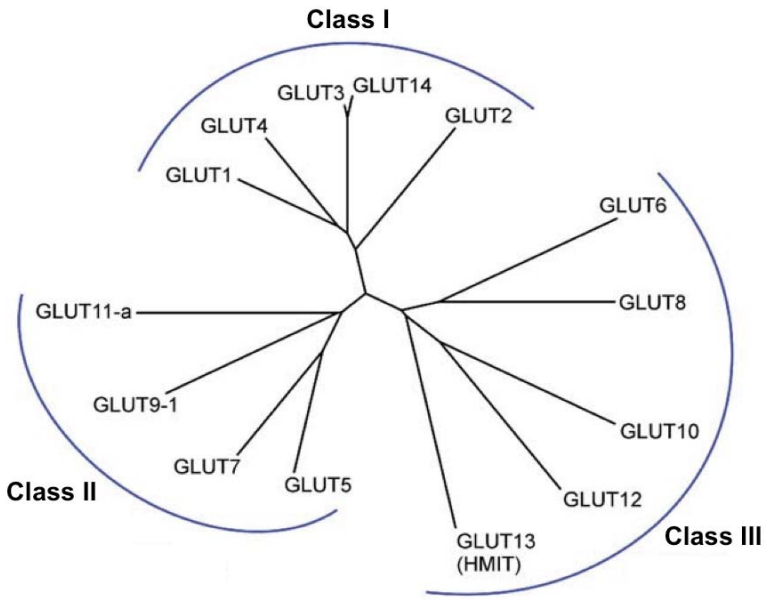
Introduction

1. Glucose transporters

Glucose is a central molecule to the metabolism in vertebrates and plays a pivotal role as fuel and metabolic substrate (Wood and Trayhurn, 2003). Glucose is obtained mainly in two ways: directly from the diet and derived from glycogen that is mainly stored in the liver (Yamashita et al., 2001; Wood and Trayhurn, 2003; Postic et al., 2007). Glucose derived from the diet is transferred from the lumen of the small intestine to the circulation and subsequently into target cells to be used as an energy source or, when in excess, to be stored as hepatic glycogen or as triglycerides (Yamashita et al., 2001; Wood and Trayhurn, 2003; Postic et al., 2007). The transfer of glucose across plasma membranes is mediated by two different groups of transporters: the Na^+-dependent glucose co-transporters (SGLT, members of a larger family of Na^+-dependent transporters, gene name SLC5A) (Wright, 2001); and by the facilitative Na+-independent sugar transporters (GLUT family, gene name SLC2A) (Mueckler, 1994; Uldry and Thorens, 2004).

The GLUT protein family belongs to the Major Facilitator Superfamily (MFS) of membrane transporters (Pao et al., 1998) being most of them able to catalyze the facilitative (energy-independent) bidirectional transfer of their substrates across membranes (Thorens and Mueckler, 2010). GLUTs are proteins composed of approximately 500 amino acids that possess 12 trans-membrane-spanning alpha helices and a single N-linked oligosaccharide (Joost and Thorens, 2001; Joost et al., 2002; Zhao and Keating, 2007; Augustin, 2010). The GLUT protein family members can be grouped into three different classes based on their sequence similarities (Fig. 1). Class I is comprised of the classical glucose transporters GLUT1 to GLUT4 and GLUT14 which is a duplication of GLUT3; class II is comprised of GLUT5, GLUT7, GLUT9, and GLUT11 and class III is comprised of GLUT6, GLUT8, GLUT10, GLUT12 and GLUT13 (proton driven myoinositol transporter HMIT) (Joost and Thorens, 2001; Joost et al., 2002; Zhao and Keating, 2007; Augustin, 2010).

A



B

Protein	Expression
Class I	**GLUT1** Placenta, brain, kidney, colon, eye, skeletal muscle, peripheral nerve and erythrocytes **GLUT2** Liver, pancreas, small intestine, kidney and brain **GLUT3** Brain, placenta and testis **GLUT4** Skeletal muscle, heart and adipose tissue **GLUT14** Testis
Class II	**GLUT5** Testis, intestine, kidney and muscle **GLUT7** Intestine, testis and prostate **GLUT9** Liver and kidney **GLUT11** Pancreas, kidney, placenta and muscle
Class III	**GLUT6** Brain, spleen and peripheral leukocytes **GLUT8** Testis **GLUT10** Liver and pancreas **GLUT12** Heart and prostate **GLUT13** Brain

Figure 1. The glucose transporter family members. A, Unrooted radial phylogenetic tree showing the relationship between the 14 human GLUT protein family members. Adapted from (Augustin, 2010). **B**, Summary of GLUT proteins indicating the tissue where each member appears expressed.

2. Class I facilitative glucose transporters

The class I of facilitative glucose transporters includes GLUT1 to GLUT4 and GLUT14, and these transporters have been extensively characterized in terms of structure, function and tissue distribution (Fig. 1 and 2). GLUT1 is expressed mainly in placenta, brain (including the blood–brain barrier), kidney, colon, eye, skeletal muscle, peripheral nerve and erythrocytes (Joost et al., 2002; Pereira and Lancha, 2004; Zhao and Keating, 2007). GLUT1 is known to play an important role supplying the cells of the central nervous system with glucose (Thorens and Mueckler, 2010). GLUT2 is expressed primarily in pancreatic β-cells, where is involved in the glucose-sensing mechanism, the liver, intestine, the kidneys and the brain (Thorens et al., 1988; Guillam et al., 2000; Joost and Thorens, 2001; Panserat et al., 2001). GLUT2 is expressed on the basolateral membrane of hepatocytes, enterocytes and proximal renal tubules allowing for the bi-directional transport of glucose under hormonal control (Thorens et al., 1990; Thorens, 1992; Wright et al., 2003) and has been shown to be able to translocate to the apical membrane from the cytosol in enterocytes and in proximal renal tubules (Kellett and Helliwell, 2000; Marks et al., 2003). GLUT3 has a high affinity for glucose and this is consistent with its presence in tissues where the demand for glucose as fuel is considerable, mainly in the brain, testicle and placenta (Shepherd et al., 1992; Haber et al., 1993; McCall et al., 1994).

The insulin-responsive glucose transporter GLUT4, together with GLUT1, represents the most intensively studied glucose transporter. It is expressed in heart, skeletal muscle and adipose tissue (Zorzano et al., 1997; Thai et al., 1998; Khayat et al., 2002; Abel, 2004; Watson et al., 2004; Valverde et al., 2005), where it is responsible for the reduction in the postprandial rise in plasma glucose levels (Huang and Czech, 2007). Insulin acts by stimulating the translocation of specific GLUT4-containing vesicles from intracellular stores to the plasma membrane resulting in an immediate increase in glucose transport (Bryant et al., 2002). Various animal and human models for insulin-resistant states, such as obesity and type II diabetes mellitus, exhibit disrupted regulation of GLUT4 suggesting a pivotal role of this transporter in insulin-resistant

pathologies (Huang and Czech, 2007). Finally, GLUT14 appears specifically expressed in testicle (Wu and Freeze, 2002) and has a high degree of similarity with GLUT3 (95% at the nucleotide level) which, together with its chromosomic localization, led Wu and co-workers to propose that GLUT14 is a gene duplicate of GLUT3 (Wu and Freeze, 2002). However, the specific role of GLUT14 in glucose metabolism in testis remains unknown to date.

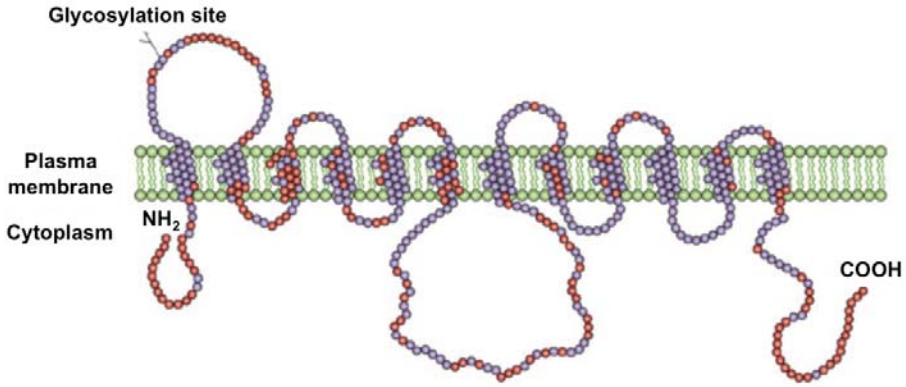


Figure 2. Schematic model for class I GLUT family members. The diagram shows the 12 trans-membrane-spanning alpha helices, with the glycosylation site located in the first extracellular loop, and the amino- and carboxy-terminus located intracellularly. Adapted from (Bryant et al., 2002).

3. GLUT2 (SLC2A2)

GLUT2 was first cloned in 1988 from human liver and kidney cDNA libraries by Thorens et al. (Thorens et al., 1988). Expression of GLUT2 was first detected in pancreas, liver, intestine and kidney (Augustin, 2010; Thorens and Mueckler, 2010) and later studies showed expression also in the brain (Leloup et al., 1994; Li et al., 2003). GLUT2 is a low-affinity, high-capacity glucose transporter with a uniquely high *Km* for glucose (17 mM) (Johnson et al., 1990), the highest among the known members of the GLUT family (Johnson et al., 1990). As a class I glucose transporter, GLUT2 is predicted to have the consensus structure characteristic of GLUTs belonging to this family (Fig. 2) with the only exception that the QLS motif in helix 7 is not present (Burant

et al., 1991; Baldwin, 1993). This motif is known to confer substrate specificity and the lack of it could explain the high affinity of this transporter for D-glucose, D-fructose and glucosamine (Burant et al., 1991; Baldwin, 1993; Uldry et al., 2002).

In mammals, GLUT2 is expressed at a very high level in pancreatic β-cells, being more abundant in the microvilli than in the basolateral membrane (Orci et al., 1989). GLUT2 was also found internalized in early endosomes and lysosomes in β-cells when protein glycosylation is affected by blocking the N-acetylglucosaminyl transferase GnT-4a (Ohtsubo et al., 2005) or when apoptosis is induced in β-cells by activation of caspase 8 (Wang et al., 2008). GLUT2 is also expressed in the basolateral and apical membranes of intestinal and kidney epithelial cells (Thorens et al., 1990; Thorens, 1992; Kellett and Helliwell, 2000; Marks et al., 2003; Wright et al., 2003) and in the sinusoidal plasma membrane and in endosomal fractions of hepatocytes (Thorens et al., 1990; Thorens, 1992; Eisenberg et al., 2005).

From a physiological point of view, the expression pattern of GLUT2, together with the ability of GLUT2 to transport different types of hexoses at a wide range of concentrations, ensures fast equilibration of glucose between the extracellular space and the cell cytosol, displaying bidirectional fluxes in and out of the cells (Leturque et al., 2005; Thorens and Mueckler, 2010). This mechanism contributes to a number of different processes (Fig. 3), such as intestinal and renal absorption of glucose, the stimulation of insulin secretion by glucose in β-pancreatic cells, the entry and output of glucose by the liver and the glucosensing capability of specific brain regions involved in the regulation of glucose metabolism and food intake (Burcelin et al., 2000; Guillam et al., 2000; Uldry and Thorens, 2004; Leturque et al., 2005; Marty et al., 2007; Eny et al., 2008).

Particularly in pancreatic β-cells, GLUT2 cell surface expression is responsible for glucose uptake, which will trigger the glucose-induced insulin secretion (Ohtsubo et al., 2005). This leads to plasma membrane depolarization through closure of ATP-dependent K^+ channels and Ca^{2+} influx to exocytose insulin granules (Henquin et al.,

2003). In β-cells, GLUT2 equilibrates extra- and intracellular glucose concentrations providing an unrestricted supply of glucose, being glucokinase the rate-limiting step (Matschinsky, 2002). Despite this, it has been reported that in β-cells engineered with different GLUT isoforms, only GLUT2 allows for normal insulin production in response to glucose stimulation (Hughes et al., 1993).

In enterocytes, it is well established that GLUT2 translocates from cytosolic GLUT2-containing vesicles to the apical membrane after sugar-rich meals (Gouyon et al., 2003; Kellett and Brot-Laroche, 2005). In response to the increase of sugar plasmatic levels, insulin is secreted by pancreatic β-cells, leading to the internalization of GLUT2 (Tobin et al., 2008). In this way, the capacity of sugar transport is regulated by GLUT2 adjusting it according to the luminal concentrations of glucose (Kellett et al., 2008). A similar phenomenon has been described in kidney proximal tubule cells of streptozotocin-treated rats in which GLUT2 translocates to the brush border membrane in response to glucose and is internalized after starvation (Marks et al., 2003).

Interestingly, GLUT2 is also internalized in the liver. In hepatocytes, GLUT2 is expressed in the sinusoidal membrane (Thorens et al., 1990) and undergoes insulin-dependent internalization in endosomal fractions (Eisenberg et al., 2005). This could be relevant to the inhibitory effect of insulin on hepatic glucose production (Girard, 2006).

Regarding expression of GLUT2 in the brain, little is known about its physiological function. Previous studies showed that GLUT2 is found in the cerebellum, brain nuclei, hypothalamic nuclei, neurons glial cells and astrocytes (Leloup et al., 1994; Nualart et al., 1999; Arluison et al., 2004; Arluison et al., 2004; Kang et al., 2004; Roncero et al., 2004; Marty et al., 2005). GLUT2 is involved in the detection of hypoglycemia in glial cells (Marty et al., 2005) and may be involved in the counterregulatory response mediated by GLUT2-dependent glucose sensing units of astrocytes and neurons (Marty et al., 2005). These evidences support the idea of the central role of GLUT2 in feeding regulation suggested by studies showing abnormal feeding behavior by blocking GLUT2

intracerebroventricularly in rats (Wan et al., 1998) or in GLUT2- null mice (Bady et al., 2006).

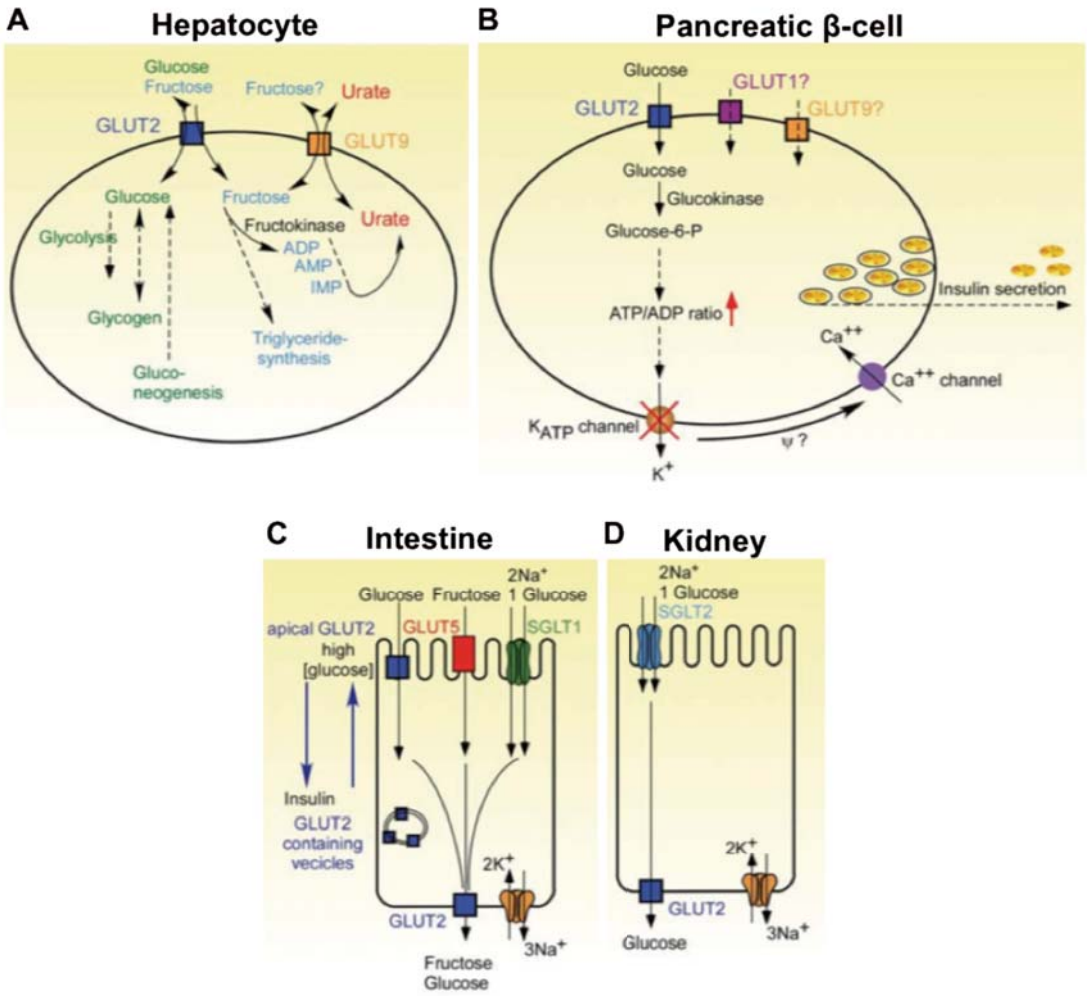


Figure 3. Physiological role of GLUT2 in different organs. A, Hexose transport in hepatocytes. GLUT2 mediates glucose uptake under feeding conditions into hepatocytes where glucose is metabolized by glycolysis or incorporated into glycogen. **B**, Pancreatic β-cells secrete insulin in response to elevations in blood glucose. GLUT2 mediates glucose uptake into β-cells leading to insulin secretion. **C**, Transepithelial glucose absorption in the intestine. GLUT2 is acutely translocated to the apical membrane of the epithelium to facilitate glucose uptake, this translocation is reversed by insulin action. **D**, Transepithelial glucose reabsorption in the kidney. Reabsorption of the glucose by GLUT2 occurs at the basolateral membrane. Adapted from Augustin (2010).

In non-mammalian vertebrates, GLUT2 has been characterized in avian and fish species (Wang et al., 1994; Krasnov et al., 2001; Hall et al., 2006; Castillo et al., 2009; Terova et al., 2009). Particularly, studies in teleost fish have shown that GLUT2 is expressed in the pancreas, liver, hindbrain and hypothalamus in rainbow trout (*Oncorhynchus mykiss*), in liver kidney and intestine in Atlantic cod (*Gadus morhua*) and in brain, heart, liver, kidney, muscle and intestine in sea bass (*Dicentrarchus labrax*) (Panserat et al., 2001; Hall et al., 2006; Polakof et al., 2007; Terova et al., 2009); while in adult zebrafish (*Danio rerio*) GLUT2 expression was found in a number of different tissues (Fig. 4) (Castillo et al., 2009). The affinity of zebrafish GLUT2 for 2-DG is 11 mM, similarly in range to the mammalian constant (Castillo et al., 2009). Moreover, zebrafish GLUT2 also allows the transport of mannose, fructose and galactose, as well as L-glucose but with low affinity, as observed in mammals (Cheeseman, 2002). These properties indicate a high degree of functional conservation between fish GLUT2 and its mammalian homolog, supporting the notion that GLUT2 is a low-affinity, high-capacity glucose transporter (Uldry and Thorens, 2004).

Regarding the physiological role of GLUT2, little is known to date in fish. It has been shown that in teleost GLUT2 expression in the pancreas and in the hindbrain is regulated by hormonal and metabolic signals (Polakof et al., 2007) whereas in the liver GLUT2 mRNA expression levels are not affected in fasting/re-feeding conditions (Panserat et al., 2001; Hall et al., 2006). In addition, hepatic mRNA levels of fish GLUT2 are up-regulated in acute- and long-term hypoxic conditions (Terova et al., 2009). Furthermore, recent studies have evidenced that GLUT2 expression in zebrafish is also under nutritional regulation in the intestine (Castillo et al., 2009).

In view of this, is evident that further studies are needed to understand the physiological role of GLUT2 and the mechanisms involved in its regulation. Lower vertebrates have been extensively recognized as a valuable tool in metabolic research (Schlegel and Stainier, 2007). In addition, there is a remarkable conservation of structure and functional characteristics of GLUT2 between fish and mammals. In this regard, future studies of the physiological role of GLUT2 in a model species such as

zebrafish will provide a valuable contribution to unravel the complex mechanisms involved in glucose homeostasis.

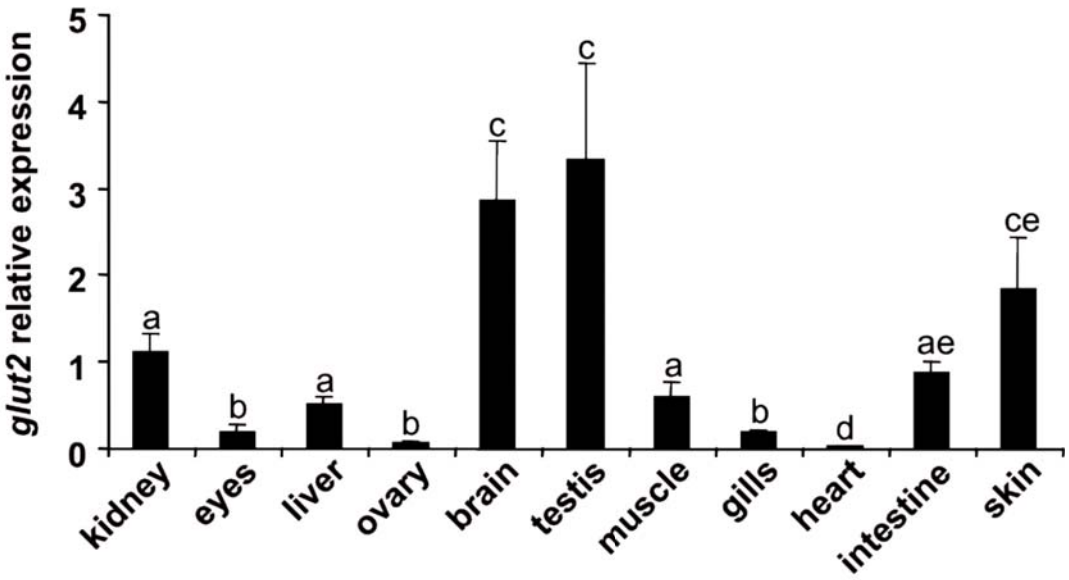


Figure 4. Tissue expression pattern GLUT2 in adult zebrafish. GLUT2 appears expressed in a broad range of tissues in adult zebrafish but especially in kidney, liver, brain, testis, muscle, intestine and skin. Adapted from (Castillo et al., 2009).

4. GLUT4 (SLC2A4)

GLUT4 was first described by James et al. in 1988 while studying glucose metabolism in tissues regulated by insulin (James et al., 1988). Soon after the discovery of GLUT4, several groups cloned it in human (Fukumoto et al., 1989), rat (Birnbaum, 1989; Charron et al., 1989) and mouse (Kaestner et al., 1989). Since its discovery, GLUT4 has received, together with GLUT1, more experimental attention than any other single membrane transport protein. Structurally, GLUT4 follows the predicted model for class I glucose transporters (Fig. 2). GLUT4 has a high affinity for glucose, with a *Km* of ≈5 mM (Huang and Czech, 2007), and also transports mannose, galactose, dehydroascorbic acid and glucosamine (Keller et al., 1989; Burant and Bell, 1992; Rumsey et al., 2000; Uldry et al., 2002).

In mammals, GLUT4 is mainly expressed in cardiac and skeletal muscle, brown and white adipose tissue, and brain (Mueckler, 1994; Rayner et al., 1994; Huang and Czech, 2007). GLUT4 plays a pivotal role in whole body glucose homeostasis, mediating the uptake of glucose regulated by insulin (Shepherd and Kahn, 1999; Saltiel and Pessin, 2002). The disruption of GLUT4 expression has been extensively associated with pathologies of impaired glucose uptake and insulin resistance such as type 2 diabetes and obesity (Kusari et al., 1991; Shepherd and Kahn, 1999; Friedel et al., 2002; Karnieli and Armoni, 2008).

Complex mechanisms regulate the expression of the GLUT4 gene since it is subjected to both tissue-specific and hormonal metabolic regulation (Knight et al., 2003). Thus, GLUT4 expression is impaired in skeletal and cardiac muscle during perinatal hypothyroidism (Castello et al., 1994; Ramos et al., 2001). This deficiency can be reverted by treatment with triiodothyronine (T_3), which increases GLUT4 mRNA levels in cardiac muscle (Castello et al., 1994). Furthermore, long-term administrations of T_3 in adult rats stimulate the expression of GLUT4 in skeletal muscle (Casla et al., 1990; Weinstein et al., 1994). Contractile activity and innervation also regulate GLUT4 expression in skeletal muscle. Previous works evidenced that GLUT4 expression is induced during the timing of skeletal muscle innervation (Castello et al., 1993) and repressed in response to muscle denervation (Block et al., 1991; Coderre et al., 1992; Castello et al., 1993). Muscle contraction also increases expression of GLUT4 in skeletal muscle. A number of studies demonstrate that GLUT4 expression is induced by electrical stimulation of muscle contraction *in vivo* and *in vitro* (Etgen et al., 1993; Hofmann and Pette, 1994; Kong et al., 1994) and after physical training in humans (Houmard et al., 1991; Dela et al., 1993) and rats (Ploug et al., 1990).

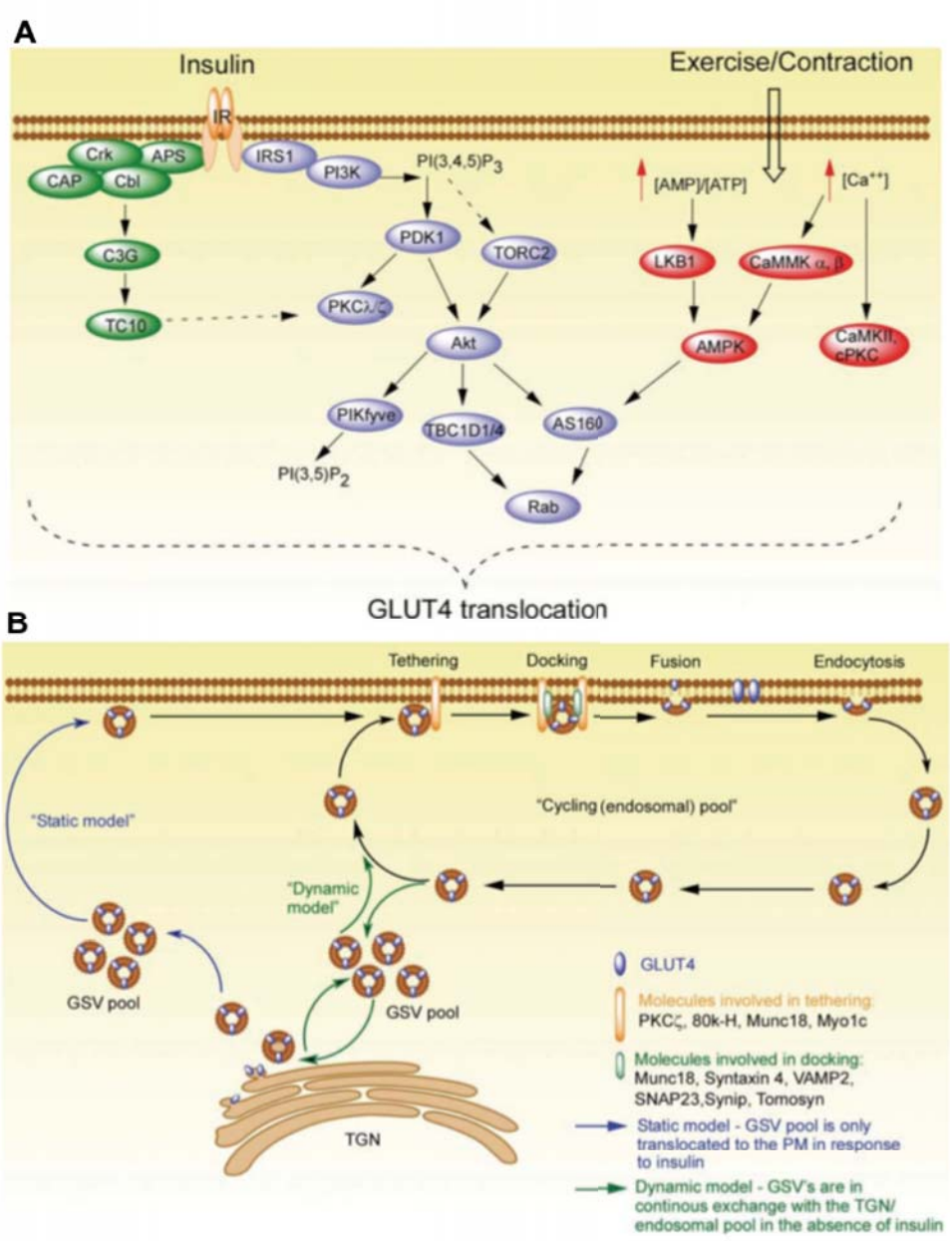


Figure 5. Schematic representation of GLUT4 regulation mechanism. A, Major signaling pathways involved in GLUT4 translocation. **B**, GLUT4 trafficking pathways. Adapted from (Augustin, 2010).

GLUT4 cycling is regulated at the levels of its exocytosis, fusion, endocytosis and inter-endosomal transit. In muscle and adipose tissue it is mainly regulated by insulin and muscle contraction (Zorzano et al., 2005; Watson and Pessin, 2006; Augustin, 2010; Lauritzen and Schertzer, 2010), allowing the entry of glucose inside the cells by

stimulating GLUT4 translocation (Fig. 5B) (Augustin, 2010). Insulin regulates the traffic of GLUT4 via phosphatidylinositol 3-kinase (PI3K) or Cbl-CAP-APS complex (Fig. 5A) (Ishiki and Klip, 2005), while muscle contraction regulates GLUT4 translocation via AMP-activated protein kinase (AMPK) (Fig. 5A) (Pereira and Lancha, 2004; Zorzano et al., 2005). In absence of insulin, GLUT4 fused to the plasma membrane is endocytosed via the early endosomal pathway and sorted into the GLUT4 storage vesicle (GSV) compartment (also called the insulin-responsive specialized compartment) involving the trans-Golgi network (TNG) (Dugani and Klip, 2005; Ishiki and Klip, 2005; Larance et al., 2008). GLUT4 is stored in the GSV pool via a static or a dynamic process, from where, in response to stimulus, will translocate to the plasma membrane (Fig. 5B) (Dugani and Klip, 2005; Ishiki and Klip, 2005; Larance et al., 2008).

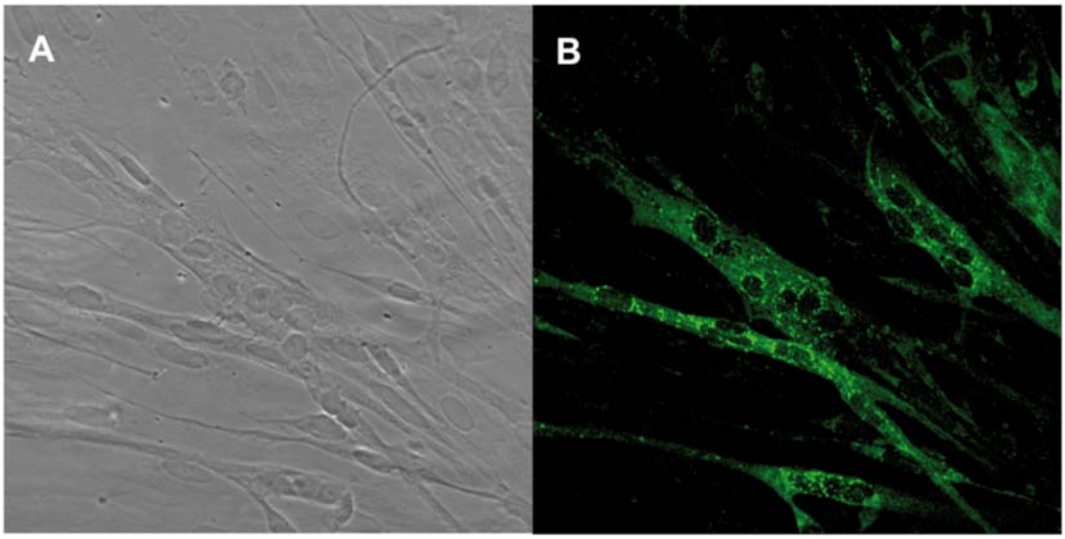


Figure 6. GLUT4 distribution in brown trout primary myotubes. A, Bright field picture of brown trout myotubes. **B**, Fluorescent picture showing the immunolocalization of endogenous brown trout GLUT4. Adapted from Diaz et al. (2007).

In non-mammalian vertebrates, GLUT4 was first characterized by Planas and co-workers in brown trout (*Salmo trutta*) red muscle (Planas et al., 2000) and, to date, it has also been cloned in other fish species (Capilla et al., 2004; Hall et al., 2006). In brown trout, GLUT4 appears highly expressed in red (Fig. 6) and white muscle, adipose tissue, kidney and gills, and more moderate expression was found in intestine and heart

(Planas et al., 2000). In other salmonid species, GLUT4 is also expressed in insulin sensitive tissues, such as in white and red skeletal muscle in rainbow trout (Capilla et al., 2002; Diaz et al., 2007; Diaz et al., 2009) and in adipose tissue in Coho salmon (*Oncorhynchus kisutch*) (Capilla et al., 2004).

Regarding the functional properties, as described in mammals, Coho salmon GLUT4 transports glucose, mannose and galactose, and, interestingly, is also able to transport fructose at high concentrations (Capilla et al., 2004). In addition, the K_m value of Coho salmon GLUT4 for glucose is ≈7.6, higher than in mammals (Capilla et al., 2004). Further kinetic analysis of 3-*O*-methylglucose transport measured under equilibrium exchange conditions showed a K_m value of 14.4 mM of Coho salmon GLUT4 (Capilla et al., 2004), again higher than the values reported in rats (Keller et al., 1989; Nishimura et al., 1993). These evidences support the notion that fish GLUTs have a lower affinity for glucose than GLUTs of higher vertebrates (Capilla et al., 2004), being consistent with the decreased ability of fish to clear glucose load compared to mammals (Moon, 2001).

Moreover, the expression of GLUT4 in fish muscle tissue is also regulated by the action of factors related to carbohydrate metabolism and glucose homeostasis. GLUT4 mRNA levels increase in response to insulin administration in brown trout (Capilla et al., 2002) and in rainbow trout red muscle (Polakof et al., 2010) and also in myoblasts and *in vitro* differentiated myotubes (Diaz et al., 2009). In the same study, Diaz et al. demonstrated that insulin- like growth factor I (IGF-I) stimulates GLUT4 expression in primary cultured cells of rainbow trout (Diaz et al., 2009), being consistent with previous data in mammals (Bilan et al., 1992). In Atlantic cod, white muscle GLUT4 increased with starvation while in the heart the expression was reduced; whereas in both tissues mRNA levels returned to pre-starved levels with re-feeding (Hall et al., 2006). Recently, a study from our group reported that 5-aminoimidiazole-4carboximide ribonucleoside (AICAR) and metformin, both activators of AMPK, increased the expression of GLUT4 in brown trout myotubes (Magnoni et al., 2012).

In addition, similarly to the mRNA, protein levels of GLUT4 in brown trout red muscle decreased significantly after fasting and increased in response to insulin administration (Diaz et al., 2007). Furthermore, in primary brown trout skeletal muscle cells, Diaz et al. demonstrated that GLUT4 translocation to the plasma membrane is stimulated in response to insulin (Fig. 7A), resulting in an increase of glucose uptake by these cells (Fig. 7B) (Diaz et al., 2007). These findings are in agreement with previous data on muscle satellite cells from rainbow trout (Castillo et al., 2004). Moreover, studies using an L6 muscle cell line stably expressing myc-labeled brown trout GLUT4 have also demonstrated that tumor necrosis factor alpha (TNFα) and AMPK activators, AICAR and metformin, significantly stimulate brown trout GLUT4 translocation to the plasma membrane (Vraskou et al., 2011; Magnoni et al., 2012). These studies also reported that stimulation with TNFα and the AMPK activators stimulated glucose uptake in trout skeletal muscle cells (Vraskou et al., 2011; Magnoni et al., 2012).

. Regarding the regulation of GLUT4 in fish adipose tissue, Capilla et al. were able to determine that brown trout and Coho salmon GLUT4, when expressed in 3T3-L1 adipocytes, translocated to the plasma membrane in response to insulin (Capilla et al., 2004; Capilla et al., 2010). Furthermore, in the same study the authors confirmed that, like in mammals, glucose uptake was increased in *Xenopus* oocytes expressing the salmon GLUT4 homolog after stimulation with insulin (Capilla et al., 2004). These results correlate with the evidence that insulin significantly stimulates glucose uptake in trout adipose tissue (Capilla et al., 2004).

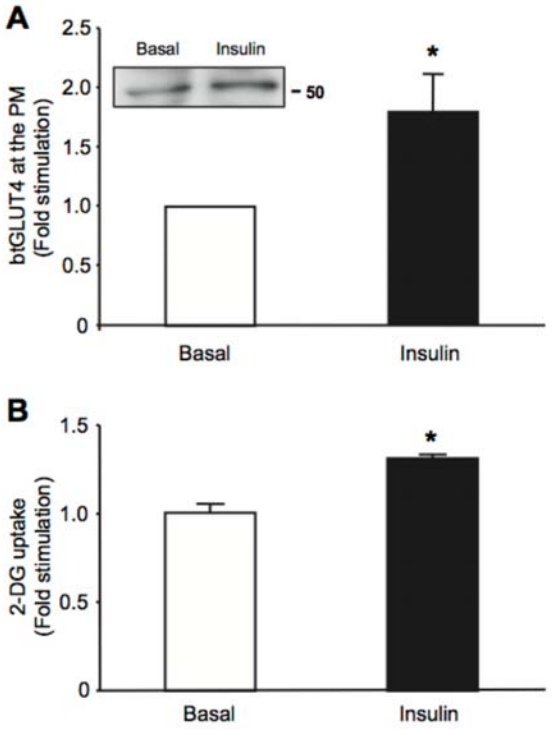


Figure 7. Effects of insulin on brown trout muscle cells. A, Endogenous brown trout GLUT4 appears increased in the plasma membrane in response to insulin administration **B**, Glucose uptake is significantly increased in cells stimulated with insulin. Adapted from (Diaz et al., 2007).

Despite these previous studies, little is known regarding the complex mechanisms taking place in the regulation and action of GLUTs and their physiological role in teleost fish metabolism. Particularly, the precise way by which insulin, one of the major molecules involved in the regulation of carbohydrate metabolism in mammals, regulates glucose metabolism in lower vertebrates has been poorly characterized to date.

In view of the need to supply further data to help decipher this complex mechanism, the objective of this thesis is to study the main glucose transporters involved in the insulin-mediated glucose metabolism, GLUT2 and GLUT4. In this light, we have studied, for the first time, the factors that govern GLUT4 gene expression in a lower vertebrate by characterizing the key elements of the transcriptional machinery in

fish using puffer fish (*Tetraodon nigroviridis*) as a model species. In addition, we have investigated the physiological role of GLUT2 in glucose metabolism in zebrafish embryos and its importance during the early stages of development. Furthermore, we have also aimed at establishing a new *in vitro* system for the study of the function of zebrafish GLUT2 by using MIN6-B1, a stable pancreatic cell line from mice.

Objectives

In this thesis we pursued the following specific objectives:

1. To study the transcriptional regulation of the GLUT4 gene in teleost fish by identifying and characterizing a functional promoter region of the Fugu (*Takifugu rubripes*) GLUT4 gene and to describe its regulation by insulin, a PPARγ agonist and contractile stimuli in skeletal muscle cells.

2. To study the localization of GLUT2 expression in zebrafish (*Danio rerio*) embryos throughout early developmental stages.

3. To evaluate the effects of abrogating zebrafish GLUT2 with antisense morpholinos on embryonic development and to describe the functional alterations caused by the GLUT2 knockdown.

4. To study the functionality of zebrafish GLUT2 using a mammalian *in vitro* model and to investigate its ability to rescue GLUT2-deficient MIN6 pancreatic cells.

Chapter I

GLUT4:
Transcriptional regulation in a non-mammalian vertebrate

"Science, like art, is not a copy of nature
but a re-creation of her"
Jacob Bronowski

1. Introduction

In mammals, the glucose transporter 4 (GLUT4) is the main facilitative glucose carrier responsible for the insulin-regulatable glucose uptake in skeletal muscle and adipose tissue (Scheepers et al., 2004). This facilitative glucose transporter exerts its function by moving to the plasma membrane from intracellular stores in response to insulin (Watson and Pessin, 2006) but also in response to muscle contraction (Lauritzen and Schertzer, 2010), allowing the entry of glucose into muscle cells. Because of this, GLUT4 has been described as the main glucose transporter responsible for insulin-mediated glucose uptake in muscle contributing to systemic glucose uptake in postprandial conditions.

The regulation of the expression of the GLUT4 gene is governed by complex mechanisms as it is subjected to both tissue-specific and hormonal metabolic regulation (Knight et al., 2003). Changes in GLUT4 expression are observed in physiological states of altered glucose homeostasis. It has been reported that the levels of GLUT4 mRNA in skeletal muscle increase with exercise training and decrease during states of insulin deficiency (Ren et al., 1994; Kawanaka et al., 1997; Host et al., 1998), and these changes are due to alterations in the transcription rate of the GLUT4 gene (Gerrits et al., 1993; Neufer et al., 1993). Therefore, unraveling the mechanisms involved in the regulation of GLUT4 transcription will assist in understanding the molecular processes regulating glucose homeostasis. In this light, previous studies have characterized several cis-acting elements regulating the transcription of the human, mouse and rat GLUT4 promoters using transgenic mice models. It has been reported that a region of 1154 bp of the 5´-flanking region of the human GLUT4 gene is essential to drive its expression in response to insulin (Olson and Pessin, 1995). The regions located within 730 bp upstream of the human GLUT4 gene (Olson and Pessin, 1995) and 522 bp upstream of the rat GLUT4 gene (Liu et al., 1994) contain motifs shown to be essential for the tissue specific expression of the GLUT4 promoter, such as binding sites for the myocyte enhancer factor 2 (MEF2).

A number of different factors appear to be involved in the transcriptional regulation of the GLUT4 gene, such as SP1, CCAAT/enhancer-binding protein (C/EBP), peroxisome proliferator-activated receptor-γ (PPARγ), hypoxia inducible factor 1a (HIF-1a), E-box, sterol regulatory element binding protein 1c (SREBP-1c), Krüppel-like factor 15 (Klf15) and nuclear factor NF1 (Zorzano et al., 2005; Karnieli and Armoni, 2008). Strikingly, little is known regarding the transcriptional activation or regulation of GLUT4 in mammals and to date there are no data in lower vertebrates. In this light, for a better understanding of the role of GLUT4 in lower vertebrates and the mechanisms involved in its regulation, we set out to identify and characterize the GLUT4 promoter in teleost fish.

In this study, we report on the identification and characterization of a functional promoter region of the Fugu (*Takifugu rubripes*) GLUT4 gene. We have determined three transcription start sites (TSSs) and confirmed the homology of this regulatory region with that of the GLUT4 gene in other teleost species. Moreover, we show how the regulatory region of the GLUT4 gene of a teleost fish presents most of the binding motifs described as important for the transcriptional regulation of GLUT4 in mammals. Moreover, we provide evidence of how the Fugu GLUT4 transcription is inhibited by insulin, and stimulated in response to a PPARγ agonist and muscle fiber contraction. Transient transfection of various 5´deletion constructs showed that the response to PG-J2 (a PPARγ agonist) depends on the number of PPARγ binding motifs and suggests the implication of HIF-1a in the regulation of the transcriptional response of the Fugu GLUT4 gene to contractile activity.

2. Materials and methods

Materials

Human insulin and 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (PG-J2) were purchased from Sigma (St Louis, MO, USA). α-MEM, fetal bovine serum (FBS) and other tissue culture reagents were purchased from Invitrogen (Prat del Llobregat, Spain).

Cloning of Fugu GLUT4 gene constructs

The firefly luciferase pGL3 expression vector system (Promega, Madison, WI, USA) was used as a reporter gene to evaluate the transcriptional activity of the 5´-flanking region of the Fugu GLUT4 gene. RT-PCR using specific primers with the sequence of the KpnI and XhoI restriction sites added (Table 1) and genomic DNA from Fugu as template, was performed under the following conditions: 3 min at 94 °C followed by 35 cycles of 94 °C for 45 s, 59 °C for 30 s and 72 °C for 1 min, and a final step of 72ºC for 10 min. The PCR product of 1314 bp was isolated from the agarose gel using Geneclean Spin Kit (MP Biomedicals, Solon, Ohio) and ligated into the pGEM-T Easy vector (Promega Corp. Madison, WI, USA).

The promoter fragment was removed from the pGEM-T Easy vector with the KpnI and XhoI restriction enzymes (New England Biolabs, MA, USA) and ligated into the pGL3-basic vector digested with the same enzymes. Following the same strategy, the 5′ deletion constructs were generated by PCR using specific primers with the sequence of the KpnI and XhoI restriction sites added (Table 1) and pGL3-FuguGLUT4 as template. All the constructs were verified by sequencing at least two times from each side with RV3 and GL2 vector primers using the BigDye v3.1 sequencing kit (Applied Biosystems, Foster City, CA). The pGL3-ratGLUT4 construct was kindly donated by Dr. Rafael Salto (University of Granada, Granada, Spain) (Giron et al., 2008).

Table 1. Primer sequences used in the cloning of the deletion constructs and in the identification of the transcription start sites.

Primer Name	Usage	Primer sequence (5´→3´)
-1072_For	-1072 Cloning	TTGCAGGTACCTTGTGCCGTGAGAGCGTCAATG
-841_For	-841 Cloning	TTGCAGGTACCTCAGATGGTGTCAAGTTCCTCCGTTC
-766_For	-766 Cloning	TTGCAGGTACCAAAGGAGGTGGCGTGATGTGGG
-608_For	-608 Cloning	TTGCAGGTACCCCTGTTGCCTGGTTGAAATGGAT
-324_For	-324 Cloning	TTGCAGGTACCTGGTCTCCACATTGGATTTGTTGAG
-132_For	-132 Cloning	TTGCAGGTACCTCTCAAGAACAGAGGCGCAGTGG
+94_For	+94 Cloning	TTGCAGGTACCTGCAGCTTGAGGTCGAGACTTGTT
pGLUT4_Rev	Cloning	TTGCACTCGAGCAGGCAGCTGCAGATGACAGAT
GSP1_Rev	GeneRacer	CCCACGCAGAAGGAGGACAACAT
GSP2_Rev	GeneRacer	CAGAGATCCCAGGACGGCGGTGAAG

Determination of the transcription start site

The TSS of Fugu GLUT4 mRNA was determined using the GeneRacer Kit (Invitrogen) following the manufacturer's indications. This technique is based on RNA ligase-mediated rapid amplification of 5´and 3´cDNA ends (RLM-RACE) and oligo-capping rapid amplification cDNA ends (RACE) methods. Nested PCR was performed using gene specific primers (GSPs) (Table 1) and total RNA isolated from Fugu dorsal muscle kindly donated by Dr. Shugo Watabe (University of Tokyo) as template. Fragments were amplified using Platinum® *Taq* DNA Polymerase (Invitrogen, Prat del Llobregat, Spain). PCR conditions were as follows: 94ºC for 3 min followed by 35 cycles of 94ºC for 45 s, 60ºC for 30 s and 72ºC for 1 min, and a final step of 72ºC for 10 min. Fragments were ligated into pCR®4-TOPO (Invitrogen, Prat del Llobregat, Spain) and transformed into One Shot® TOP10 competent cells (Invitrogen, Prat del Llobregat, Spain).

Sequence analysis

The promoter sequences were aligned and their conservation profiles were analyzed with zPicture software (zpicture.dcode.org (Ovcharenko et al., 2004)). Analysis

of the sequences for transcription factor binding sites was conducted with MatInspector (www.genomatix.com (Quandt et al., 1995)) and AliBaba 2.1 softwares (www.gene-regulation.com/pub/programs/alibaba2; (Grabe, 2002)). CpG island mapping was performed using MethPrimer software (www.urogene.org/methprimer; (Li and Dahiya, 2002)).

Cell strains and cell culture conditions

The rodent derived skeletal muscle cells lines L6 (rat) and C2C12 (mouse) were maintained with α-MEM containing 10% FBS and 1% antibiotic-antimycotic solution (10,000 U/ml penicillin G, 10 mg/ml streptomycin, 25 µg/ml amphotericin B) in an atmosphere of 5% CO_2 at 37 ºC. At confluence, C2C12 myoblasts were induced to differentiate into myotubes by culturing the cells for at least 5 days in α-MEM with the percentage of FBS reduced to 2%.

Transient transfection and promoter activity measurements

Approximately 2.5 x 10^5 cells/well were plated in 12 well dishes in the case of C2C12 cells and 10^5 cells/well were plated in 24 well dishes in the case of L6 cells and transfected 24 hours later at a confluence of 80-90% with Lipofectamine 2000 Invitrogen (Prat del Llobregat, Spain) following the manufacturer's indications. The reporter construct was co-transfected with pRL-TK (renilla) plasmid as internal standard at a 10:1 reporter construct/pRL-TK ratio. After stimulation, cells were lysed for luciferase activity measurement. Promega's Dual-Luciferase reporter system was used according to the manufacturer's instructions. Measurements were performed with an Infinite® M200 reader (Tecan Trading AG, Switzerland). The RLU of firefly luciferase was normalized against the RLU of the internal renilla luciferase standard.

Electrical stimulator settings

Transiently transfected C2C12 myotubes in 12 well dishes were subjected to the Electrical Stimulator of Cultured Cell System (ESCC), as described by Marotta and co-workers (Marotta et al., 2004). The electrical pulse stimulations were carried out inside a cell incubator for 90 min at 37 ºC in an atmosphere of 5% CO_2, at 40 V with a pulse duration of 30 ms and a frequency of 3 Hz.

Statistical analysis

Statistical analyses were performed using SPSS11 (SPSS, Chicago, IL). Statistical differences between luciferase activities were analyzed by Mann-Whitney non-parametric test and were considered to be significant at $P < 0.05$.

3. Results

Cloning of Fugu GLUT4 promoter

By searching for a DNA sequence containing the fish GLUT4 promoter, a 1.3 kb genomic sequence upstream of the *slc2a4* (GLUT4) gene was retrieved from the Fugu genomic sequence database in Ensembl (www.ensembl.org). Using sequence-specific primers (Table 1), a DNA fragment of 1,314 bp was amplified, starting 8 bp ustream of the Fugu GLUT4 ATG. This 1.3 kb fragment was cloned into the pGL3 luciferase reporter vector and confirmed by sequencing.

Identification of Fugu GLUT4 transcription start sites

We determined the TSSs of the Fugu GLUT4 gene by 5´RACE. This yielded three PCR product sizes of 452, 307 and 261 bp (Fig. 1). Analysis of these sequences

demonstrated that the PCR products corresponded to three different transcripts starting at -145, +1 and +47, respectively. The high presence of the 307 bp transcript, consistent with the observed basal activity of the different Fugu GLUT4 promoter deletion constructs (see below), led us to denote this as +1 and, consequently, as the main TSS in Fugu skeletal muscle.

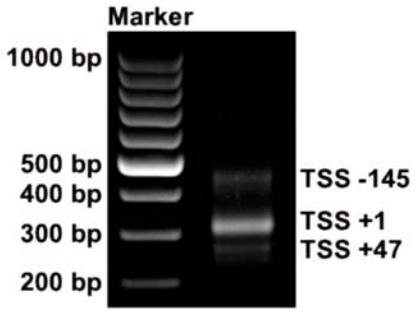


Figure 1. Determination of the transcription start sites by 5´RACE. Agarose gel electrophoresis of nested PCR reaction products from the 5´ RACE yielded three product sizes of 452, 307 and 261 bp and were denoted as -145, +1 and +47 respectively.

Fugu GLUT4 promoter sequence analysis

In silico analysis of the cloned 1.3 kb 5´-flanking region of the Fugu GLUT4 gene revealed the presence of multiple putative binding sites for transcription factors such as MEF2, SREBP, KLF, SP1/GC-box, NF-Y, E-box, PPAR-γ, PPAR-RXR and HIF-1 (Fig. 2). However, this analysis also demonstrated that this region lacked identifiable TATA boxes. In this light, we searched for CpG islands which, together with the multiple predicted SP-1 transcription factor binding sites, are characteristic of the TATA-less promoters (Anish et al., 2009, Zhu et al., 2008). Using MethPrimer software (http://www.urogene.org/methprimer/(Li and Dahiya, 2002)) it was determined that the cloned genomic region of the Fugu GLUT4 gene contains a CpG island of 260 bases, between -234 and +93 nucleotides, with 163 CG dinucleotides (Fig. 3).

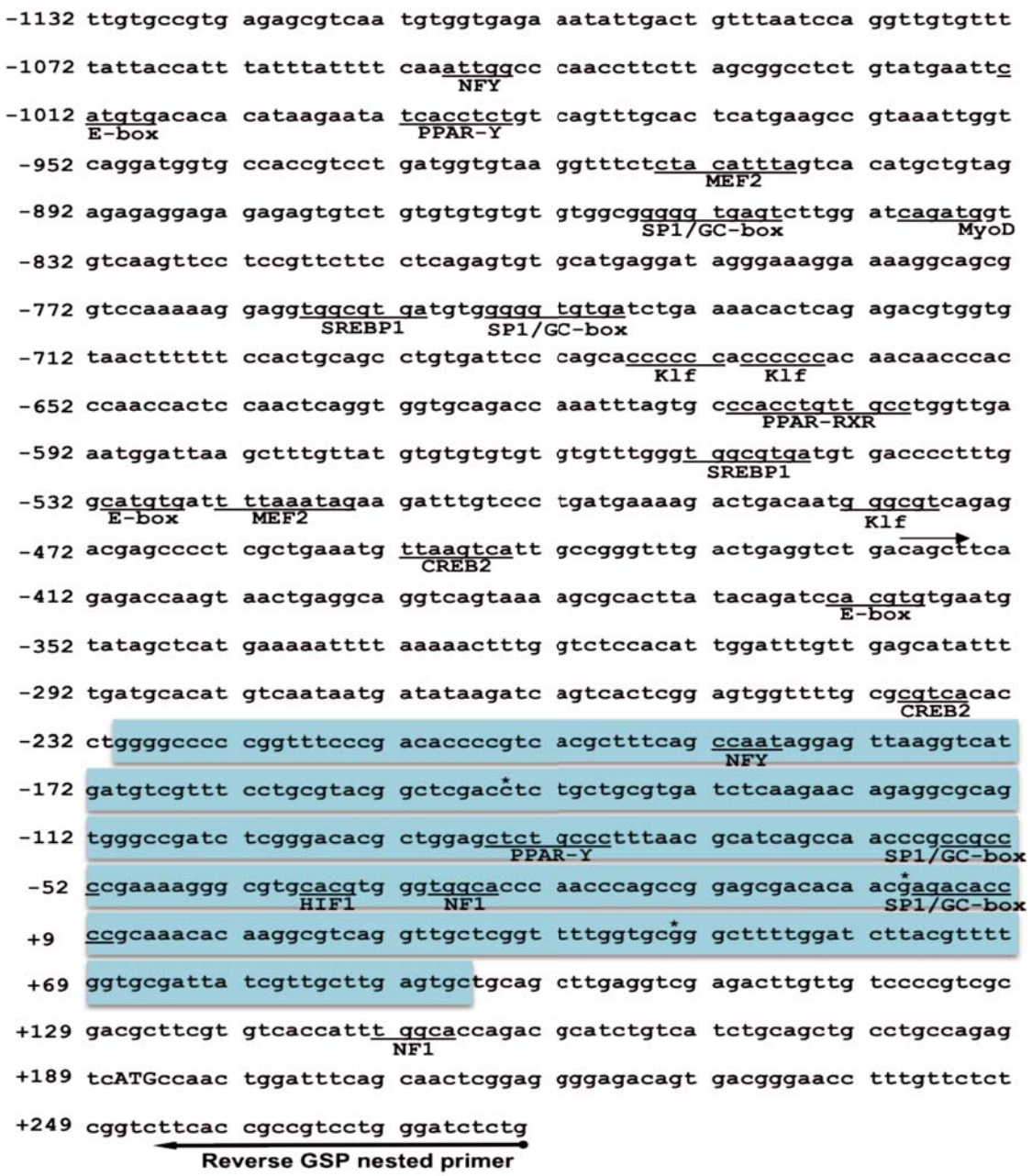


Figure 2. Analysis of the 5´ flanking region of the Fugu GLUT4 gene. Sequence of the -1132/+277 Fugu GLUT4 promoter sequence. The three transcriptions start sites are indicated with an asterisk (*). Positions are given relative to the major transcription start site assigned with the +1 position. The translation start codon ATG is indicated with capital letters. Putative binding sites for transcription factors are underlined. Promoter specific reverse GSP nested primer is underlined with an arrow. Blue boxes highlight the predicted CpG island.

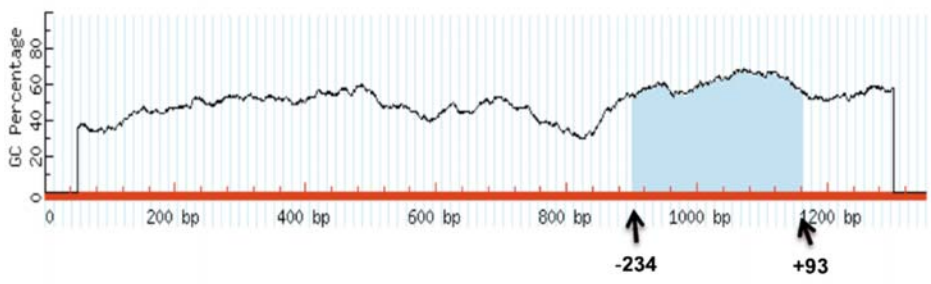


Figure 3. Prediction of a CpG island (blue area) within the 5´ cloned region of the Fugu GLUT4 gene. Black arrows indicate the positions delimiting the CpG island relative to the +1 TSS.

Next, we examined the conservation profile of the available teleost fish genomic sequences from databases corresponding to the 1.3 kb genomic DNA upstream to the GLUT4 gene using zPicture software. Sequence comparison between tetraodon (*Tetraodon nigroviridis*) and Fugu showed two major regions, between -786/-334 and -234/+182 nucleotides, that were highly conserved, with a similarity higher than 70%, (Fig. 4). A similar conserved sequence profile was observed between stickleback (*Gasterosteus aculeatus*) and Fugu, with similarities between 50% and 70% in the conserved regions (Fig. 4). Despite the lack of significant similarities between the medaka (*Oryzas latipes*) and Fugu GLUT4 promoters, comparison between medaka and stickleback sequences confirmed that the GLUT4 promoters of all species analyzed contain two conserved areas, one of which surrounds the TSS+1 (Fig.4). Overall, these results indicate that the regions comprised between -786/-334 and -234/+182 nucleotides contain most of the relevant predicted binding motifs involved in the regulation of the Fugu GLUT4 gene, as well as the TSS+1.

Identification of the transcriptional regulatory regions of the Fugu GLUT4 gene

To verify the functionality of the cloned Fugu GLUT4 promoter, we first transiently transfected the luciferase construct containing the 1.3 kb DNA fragment (from now on denoted as -1132) into L6 cells. In parallel, a similar luciferase construct containing the rat GLUT4 promoter was transiently transfected as a control for the activity of the cloned

Fugu GLUT4 promoter. These experiments indicated that the Fugu GLUT4 promoter was functional and that its basal activity was significantly higher compared to that of the mammalian GLUT4 promoter (Fig. 5A).

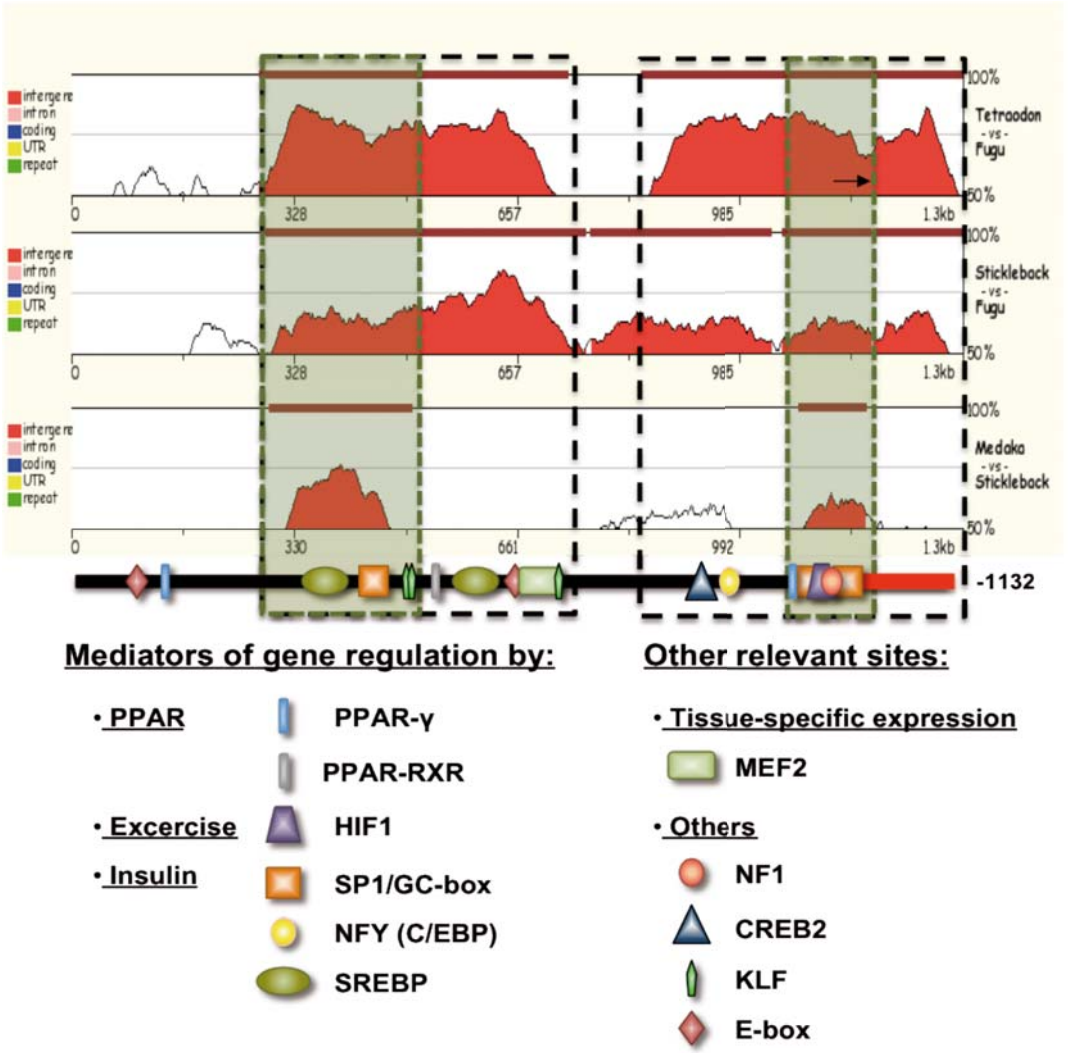


Figure 4. ECR Browser conservation profile of the 5´ region of the Fugu GLUT4 gene. Sequence elements of significant length (≥ 100 nucleotides) that present a sequence homology higher than 60% are highlighted in red and depicted with the dark-red rectangles on the top of the graph. The horizontal axis represents the position of the nucleotides within the 1314 bp sequence. The vertical axis represents the percent of identity between the aligned genomes. In the bottom we show a schematic representation of the -1132 Fugu GLUT4 gene promoter that highlights the most relevant predicted binding sites.

To further characterize the promoter regions of the Fugu GLUT4 promoter that are responsible for its basal activity, we generated a set of six luciferase reporter gene constructs containing consecutive deletions of the Fugu GLUT4 -1132 construct (Fig. 5B) and we transiently transfected them into L6 cells. When compared to the -1132 construct, the -901 and -826 constructs showed significantly higher transcriptional activity, whereas the -669, -383 and -132 constructs appeared to be similar to the -1132 construct and the +94 construct presented the lowest promoter activity with transcriptional levels similar to the empty vector (Fig. 5B). Transcriptional levels shown by both -901 and -826 constructs suggest the presence of a negative regulatory region upstream, between the -1132 and -826 nucleotides, and a positive regulatory region within this region, between -901 and -826 nucleotides.

These results demonstrated that the region comprised between -132 and +94 contains essential elements required for the basal activity of the Fugu GLUT4 promoter. This in consistent with the *in silico* predictions which identified PPAR-γ, HIF1 SP1/GC-box and NF1 transcription factors binding sites as well as a CpG island within this region where the TSS+1 is located. Furthermore, these results strongly suggest that the core sequence of the basal promoter is localized in the region -132 to +94.

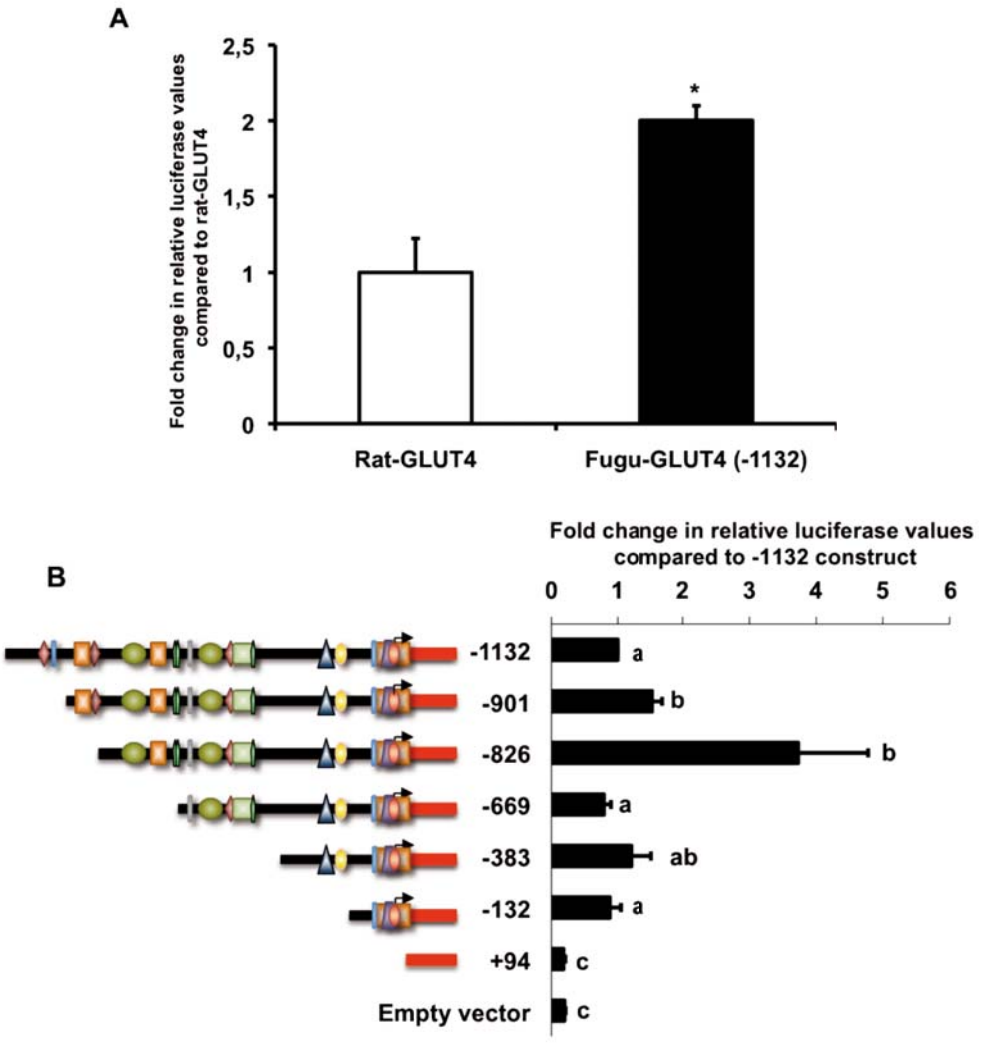


Figure 5. Activity of the Fugu GLUT4 promoter in transiently transfected L6 cells. A) Basal activity of the rat GLUT4 (Rat-GLUT4) and Fugu GLUT4 (Fugu-GLUT4) promoters transiently transfected into L6 muscle cells. Data are normalized to the relative expression of Renilla luciferase activity, setting the activity of the rat GLUT4 promoter to 1. Data are shown as fold change in activity compared to Rat-GLUT4 vector and expressed as mean ± S.E. of triplicate independent experiments. * indicates statistical significant differences ($p<0.05$). **B)** Basal activity of Fugu GLUT4 promoter deletion constructs transiently transfected into L6 muscle cells. Data are normalized to the relative expression of Renilla luciferase activity, setting the level of the different deletion constructs relative to the -1132 basal activity. Data are shown as fold change in activity compared to the -1132 promoter and expressed as mean ± S.E. of at least three independent experiments. Different letters indicate statistical significant differences between deletion constructs ($p<0.05$).

Regulation of the activity of the Fugu GLUT4 promoter by Insulin

In order to study whether insulin exerts a regulatory effect on GLUT4 expression at the level of transcription, we transiently transfected L6 myoblasts with the rat and Fugu GLUT4 promoters and stimulated the transfected cells with human recombinant insulin (100 nM) for 18 h. Insulin treatment decreased the activity of the rat and Fugu GLUT4 promoters by approximately 50% (Fig. 6), confirming the previously described effect of insulin on the rat GLUT4 promoter activity in L6 myoblasts and myotubes (Giron et al., 2008) as well as on the mouse GLUT4 promoter in 3T3-L1 adipocytes (Cooke and Lane, 1998; Cooke and Lane, 1999).

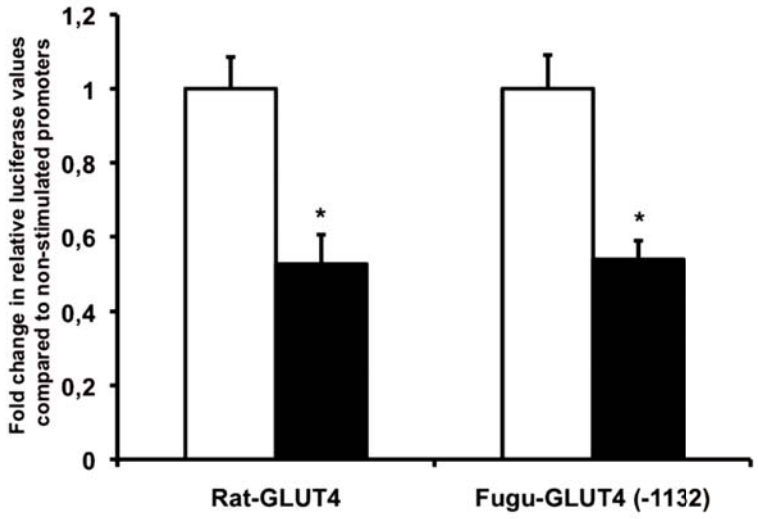


Figure 6. Activity of the rat and Fugu GLUT4 promoters in transiently transfected L6 cells stimulated with insulin. Rat and Fugu GLUT4 promoters were transiently transfected into L6 muscle cells and stimulated with human recombinant insulin (100 nM) for 18 hr. Data are normalized to the relative expression of Renilla luciferase activity, setting the activity of the non-stimulated promoter to 1. Data are shown as fold changes in activity compared to the non-stimulated promoter and expressed as mean ± S.E. of at least three independent experiments. * indicates statistical significant differences with respect to the non-stimulated promoter ($p<0.05$).

We next performed dose-response and time-course experiments in the absence or presence of insulin at concentrations of 1, 10 and 100 nM during 18 h (Fig. 7) and at different times (from 0.5 to 18 h) at a concentration of 100 nM (Fig. 8). Insulin appeared to reduce the activity of the Fugu GLUT4 promoter in a dose- and time-dependent manner. Specifically, the activity of the Fugu GLUT4 promoter was significantly reduced with respect to the control at 10 and 100 nM insulin and at 4, 8 and 18 h in the dose-response and time-course experiments, respectively.

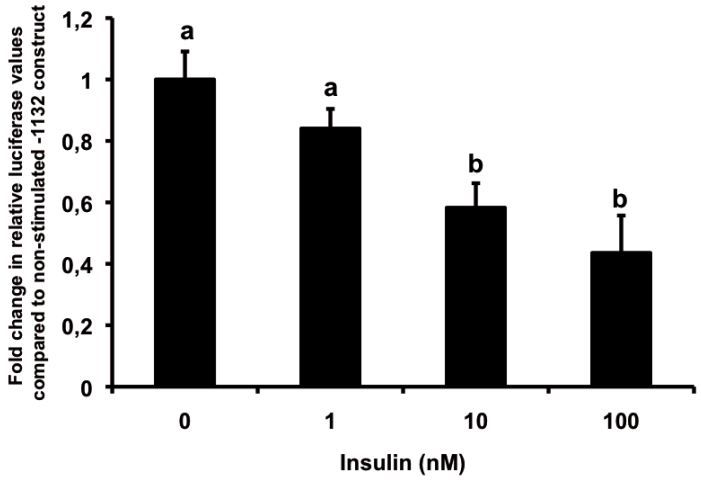


Figure 7. Activity of the Fugu GLUT4 promoter transiently transfected into L6 muscle cells in response to different insulin concentrations (1, 10 and 100nM) after 18 hours. Data are normalized to the relative expression of Renilla luciferase activity, setting the activity of the non-stimulated promoter to 1. Data are shown as fold changes in activity compared to the non-stimulated promoter and expressed as mean ± S.E. of at least three independent experiments. Different letters indicate statistical significant differences ($p<0.05$).

To further characterize the regions potentially involved in the regulation of the activity of the Fugu GLUT4 promoter by insulin, we transiently transfected L6 myoblasts with the various deletion constructs and incubated the cells (Fig. 9) for 18 h in the absence or presence of insulin (100 nM). All the six constructs generated showed a significant reduction in their transcriptional activity in response to insulin.

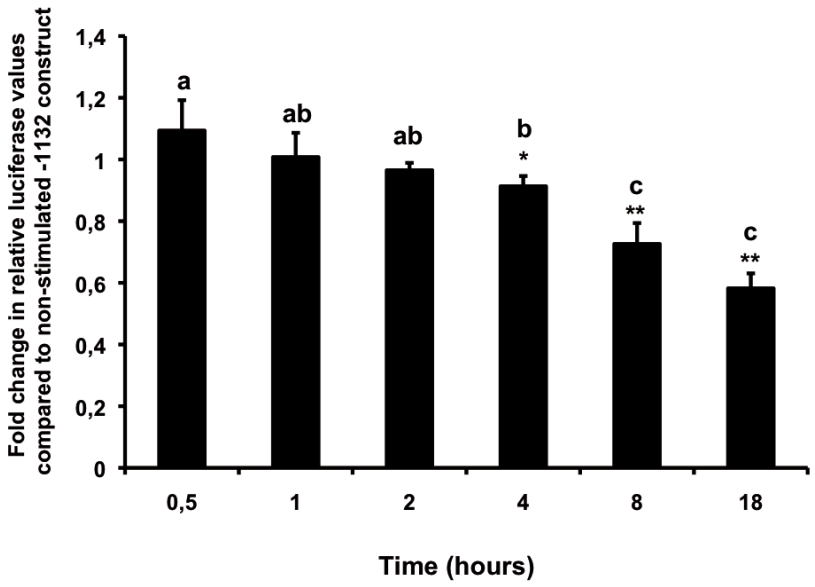


Figure 8. Activity of the Fugu GLUT4 promoter transiently transfected into L6 muscle cells at different time points (0,5, 1, 2, 4, 8 and 18 hours) in the absence or presence of insulin (100nM). Data are normalized to the relative expression of Renilla luciferase activity, setting the activity of the non-stimulated constructs to 1. Data are shown as fold changes in activity compared to the non-stimulated constructs and expressed as mean ± S.E. of at least three independent experiments. * indicates statistical significant differences with the non stimulated constructs ($p<0.05$). ** indicates statistical significant differences with the non stimulated constructs ($p<0.01$). Different letters indicate statistical significant differences between the stimulated constructs ($p<0.05$).

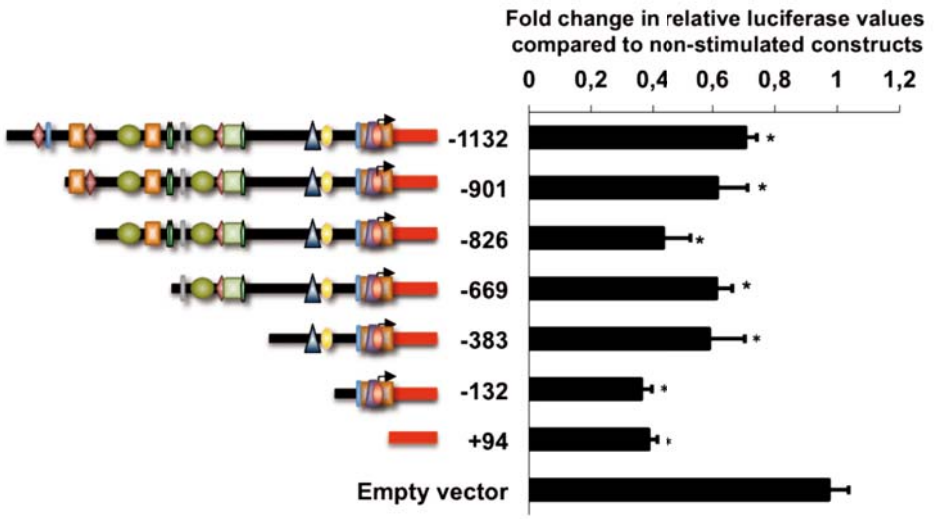


Figure 9. Activity of the Fugu GLUT4 promoter deletion constructs transiently transfected into L6 muscle cells in the absence or presence of insulin (100 nM) after 18 hours. Data are normalized to the relative expression of Renilla luciferase activity, setting the activity of the non-stimulated constructs to 1. Data are shown as fold changes in activity compared to the non-stimulated constructs and expressed as mean ± S.E. of at least three independent experiments. * indicates statistical significant differences with the non stimulated constructs ($p<0.05$).

Regulation of the activity of the Fugu GLUT4 promoter by PPARγ agonists

To address if PPARγ is involved in the regulation of the GLUT4 gene at the transcriptional level, we tested the effects of PG-J2, a natural PPARγ ligand. For this purpose, we stimulated L6 myoblasts that were transiently transfected with the -1132 construct in the absence or presence of PG-J2 (10 μM) for 18 h. Interestingly, treatment with PG-J2 increased significantly the transcriptional activity of the Fugu GLUT4 promoter (Fig.10). To identify the regions involved in the regulation of the Fugu GLUT4 gene transcription by the PPARγ ligand, we transiently transfected L6 myoblasts with the -826, -383 and +94 deletion constructs and incubated them in the absence or presence of PG-J2 (10 μM) for 18 h. The -826 and -383 constructs showed a significant increase in their transcriptional activity in response to PG-J2 whereas the +94 deletion construct

did not show any change in response to PG-J2 (Fig. 11). Interestingly, we observed a relationship between the number of PPARγ binding motifs and the response to PG-J2. Sequential deletions of the PPARγ binding motifs in the full GLUT4 promoter induced a progressive reduction of the stimulatory effects of PG-J2, as shown by the significant reduction of the activity of the -383 construct when compared with that of the -1132 construct (Fig.11).

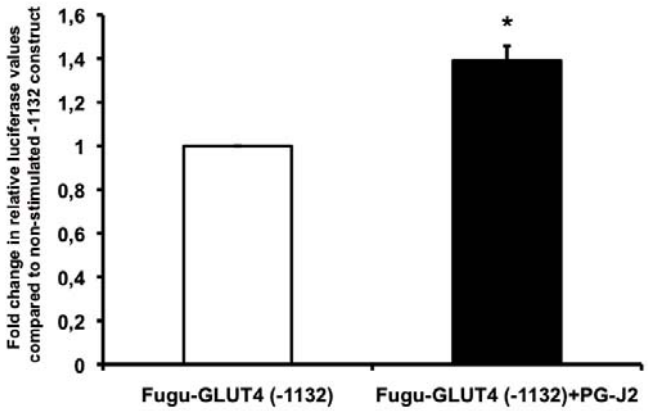


Figure 10. Activity of the Fugu GLUT4 promoter in response to ligand-induced PPARγ activation. The -1132 Fugu GLUT4 promoter construct was transiently transfected into L6 muscle cells and stimulated with PG-J2 (10 μM) for 18 hr. Data are normalized to the relative expression of Renilla luciferase activity, setting the activity of the non-stimulated promoter to 1. Data are shown as fold change in activity compared to the non-stimulated promoter and expressed as mean ± S.E. of at least three independent experiments. * indicates statistical significant differences ($p<0.05$).

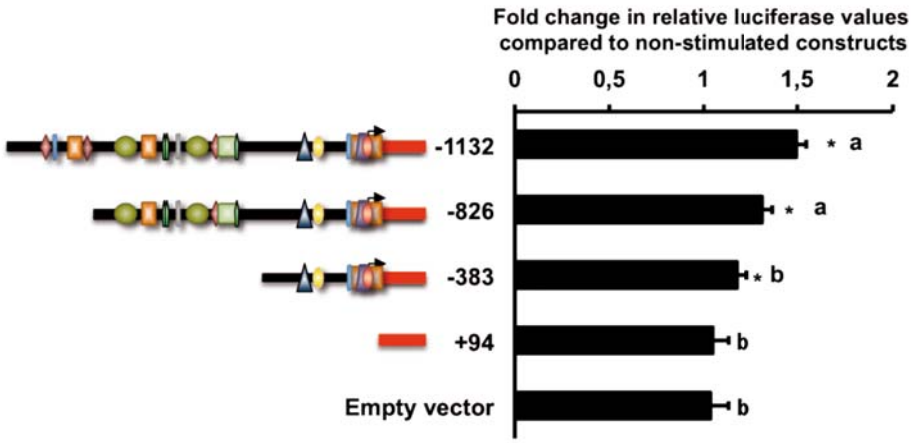


Figure 11. Activity of Fugu GLUT4 promoter deletion constructs transiently transfected into L6 muscle cells in absence and presence of PG-J2 (10 μM) after 18 hours. Data are normalized to the relative expression of Renilla luciferase activity, setting the activity of the non-stimulated constructs to 1. Data are shown as fold changes in activity compared to the non-stimulated constructs and expressed as mean ± S.E. of at least three independent experiments. * indicates statistical significant differences with the non stimulated constructs ($p<0.05$). Different letters indicate statistical significant differences between the stimulated constructs ($p<0.05$).

Regulation of the activity of the Fugu GLUT4 promoter by electrical pulse stimulation

To assess whether contractile activity regulates GLUT4 transcriptional activity, we induced contractile activity in C2C12 myotubes by electrical pulse stimulation in order to mimic exercise conditions. C2C12 myotubes transiently transfected with the -1132 Fugu GLUT4 promoter construct that were stimulated electrically had significantly higher levels of activity of the Fugu GLUT4 promoter than non-stimulated cells (Fig. 12).

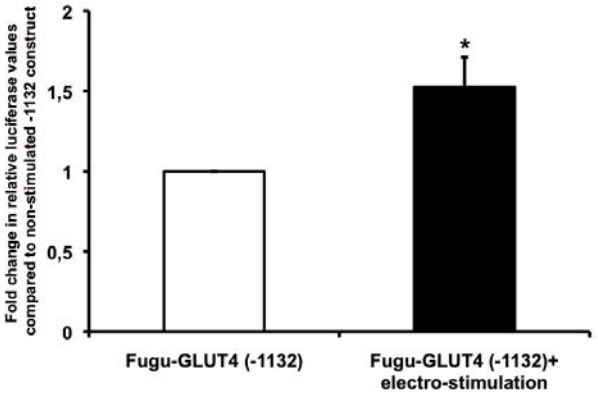


Figure 12. Activity of the Fugu GLUT4 promoter in response to *in vitro* contraction induced by electrical pulse stimulation. The -1132 **F**ugu GLUT4 promoter construct was transiently transfected into C2C12 muscle cells and electrically stimulated as described in Materials and Methods. Data are normalized to the relative expression of Renilla luciferase activity, setting the activity of the non-stimulated promoter to 1. Data are shown as fold change in activity compared to the non-stimulated promoter and expressed as mean ± S.E. of at least three independent experiments. * indicates statistically significant differences ($p<0.05$).

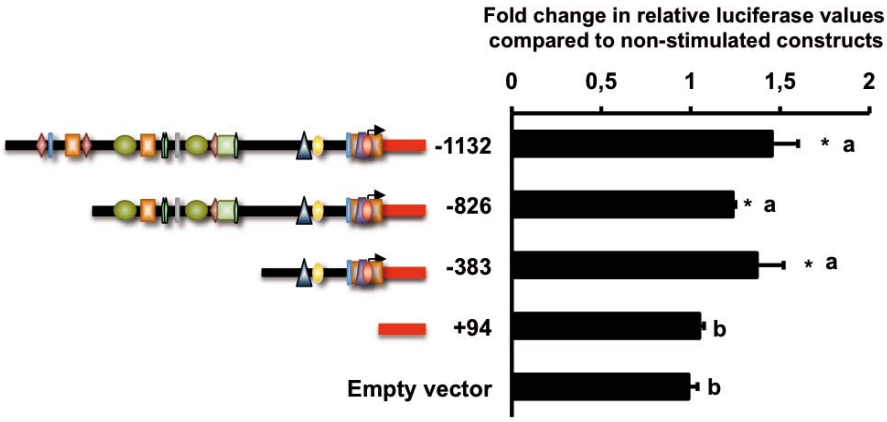


Figure 13. Activity of Fugu GLUT4 promoter deletion constructs transiently transfected into C2C12 muscle cells in the absence or presence of electrical pulse stimulation. Data are normalized to the relative expression of Renilla luciferase activity, setting the activity of the non-stimulated construct to 1. Data are shown as fold change in activity compared to the non-stimulated constructs and expressed as mean ± S.E. of at least three independent experiments. * indicates statistical significant differences with the non stimulated constructs ($p<0.05$). Different letters indicate statistical significant differences between the stimulated constructs ($p<0.05$).

Additionally, in order to characterize regions potentially involved in the regulation of the Fugu GLUT4 gene transcription by contractile activity, we studied its effects on C2C12 myotubes transiently transfected with the -826, -383 and +94 deletion constructs. Interestingly, the activity of the -826 and -383 Fugu GLUT4 deletion constructs was significantly up-regulated by electrical stimulation, while the +94 deletion construct showed no changes in its transcriptional activity, confirming it as the deletion construct that is missing the core promoter sequence (Fig. 13).

4. Discussion

In the present study, we have cloned the first functional GLUT4 promoter in a non-mammalian species. The 5´-flanking region of the Fugu GLUT4 gene contains 3 TSSs, a conserved cluster of CpG dinucleotides, three SP1 binding motifs and lacks TATA-box elements. These findings are consistent with previous reports indicating that the GC-rich promoter regions are typically characterized by the presence of multiple TSSs, the lack of TATA elements and the presence of multiple SP1 transcription factor binding sites (Brandeis et al., 1994; Macleod et al., 1994; Zhu et al., 2008; Anish et al., 2009). Interestingly, this correlates with the structure of the mammalian GLUT4 promoters that present multiple TSSs, lack TATA-box elements, contain binding sites for several nuclear transcription factors including SP1 and C/EBP and present GC-rich regions close to the core promoter (Kaestner et al., 1990; Buse et al., 1992; Liu et al., 1992; Richardson and Pessin, 1993). By comparing the structure of the cloned Fugu GLUT4 promoter with that of other vertebrate species, we have confirmed that there are two highly conserved regions that contain most of the binding motifs potentially involved in the transcriptional regulation of the GLUT4 gene (Fig. 2). Within these regions, the E-box/MEF2/Klf cassette, located between nucleotides -531 and -478, and the core promoter appear strongly conserved in all the analyzed species (Fig. 2). Similarly in the rat, mouse and human GLUT4 promoters there is a highly conserved region containing this cassette as part of a well-characterized enhancer region (Zorzano et al., 2005). It is

known that the Klf15 and E-box binding factors exert a synergistic effect on MEF2 (Zorzano et al., 2005), which is an essential binding site for the tissue-specific expression of GLUT4 in mammals (Liu et al., 1994; Olson and Pessin, 1995; Thai et al., 1998).

Next, we determined *in vitro* that the cloned promoter was capable of driving the expression of the luciferase gene when expressed in the rat muscle cell line L6, thus demonstrating the functionality of the Fugu GLUT4 promoter. Furthermore, the basal activity of the Fugu GLUT4 promoter was 2-fold higher than that of the rat GLUT4 promoter. By generating a series of 5´-deletion constructs of the Fugu GLUT4 upstream regulatory region, we confirmed that the +1 TSS as well as the presumptive core promoter were indeed located between nucleotides -132 and +93, matching completely the position of the predicted CpG island (Fig. 3). The -901 and -826 constructs were the only deletions showing a significant increase in basal activity over that of the -1132 promoter construct. This finding suggests the existence of an enhancer region between nucleotides -901 and -669. One possible explanation could be the presence of a tandem of binding sites for Klf15 between nucleotides -665 and -677. In mammals, Klf15 strongly induces GLUT4 transcription by synergizing with MEF2 (Gray et al., 2002).

It is well accepted that the phosphatidylinositol 3-Kinase (PI3K)-dependent pathway is one of the major mechanisms involved in the trafficking of GLUT4 and is required for the insulin-dependent increase in the levels of GLUT4 at the cell surface (Foster et al., 2001). Additionally, insulin has been widely described as an effective inducer of GLUT4 mRNA and protein expression as well as of GLUT4 translocation in fish (Capilla et al., 2004; Diaz et al., 2007), as in mammals (Bryant et al., 2002; Ishiki and Klip, 2005; Larance et al., 2008). Strikingly, the effect of insulin on the regulation of the transcription of the GLUT4 gene has not been fully characterized to date. Cooke and Lane observed that insulin caused a repression of GLUT4 transcription that was mediated by NF1 in adipocytes (Cooke and Lane, 1999). More recently, insulin has been shown to inhibit GLUT4 transcription in mammalian muscle cells (Giron et al.,

2008). In the present study, we have confirmed the insulin-induced repression of the rat GLUT4 promoter, used as a control for the activity of the Fugu GLUT4 promoter. Interestingly, we have also observed that the activity of the Fugu GLUT4 promoter is inhibited by insulin in a dose- and time-dependent manner. In view of the known stimulatory effects of insulin on GLUT4 mRNA levels in skeletal muscle in fish and mammalian species, the inhibition of the transcriptional activity of the GLUT4 promoter is difficult to explain. One possibility is that the regions in the GLUT4 promoter that may mediate its transcriptional activation may have been located upstream of the cloned GLUT4 promoters examined (i.e. 1.3 kb for the Fugu gene and 2.3 kb for the rat gene). Another possibility to try to explain the discrepancy between the effects of insulin on GLUT4 mRNA levels and GLUT4 promoter activity is that prolonged treatment of L6 muscle cells in the presence of insulin may have induced insulin resistance. However, the minimal effective insulin concentrations (10 nM) and incubation times with insulin (4 h) that lead to the decrease in the activity of the Fugu GLUT4 promoter are difficult to reconcile with the idea that insulin may be causing an insulin resistance phenotype in L6 cells. Interestingly, in the case of the Fugu GLUT4 promoter, all the promoter deletions, including the +94 construct, were repressed by insulin, suggesting that the promoter region that is downstream of the TSS may contain the necessary elements for mediating the repression of the GLUT4 gene. Further studies will be needed to identify the upstream regions responsible for the activation of GLUT4 promoter activity by insulin.

Next, in order to study other mechanisms potentially involved in the regulation of the Fugu GLUT4 promoter activity, we investigated the in vitro effects of PPARγ activation and experimentally controlled contraction of mammalian muscle cells expressing the Fugu GLUT4 promoter construct. Specifically, we stimulated L6 cells transiently transfected with the -1132 construct with PG-J2, an endogenous PPARγ agonist, and we also stimulated C2C12 cells transiently transfected with the -1132 construct with electrical pulse stimulation in order to induce contractile activity. Both stimuli resulted in an increase in the Fugu GLUT4 promoter activity. PPARs are ligand–activated transcription factors from the nuclear receptor family. Three PPAR isoforms (α,

β and γ) have been described, and they differ in their tissue distribution and ligand specificity (Schoonjans et al., 1997). In particular, PPARγ is involved in the regulation of lipid metabolism and glucose homeostasis (Olefsky and Saltiel, 2000) and is expressed in adipose and muscle tissue (Fajas et al., 1997). Furthermore, PPARγ has been shown to repress GLUT4 promoter activity in adipocytes (Armoni et al., 2003), while treatment with synthetic PPARγ agonist agents called thiazolidinediones (TZD) in obese Zucker fa/fa rats increased GLUT4 mRNA levels (Hallakou et al., 1997). PG-J2 has been shown to be the most potent natural ligand of PPARγ (Forman et al., 1995; Kliewer et al., 1995). Our results confirm that transcription of the GLUT4 gene is significantly activated by PG-J2, decreasing gradually with the ablation of the PPAR/RXR motifs. This is consistent with the fact that PPARγ receptors heterodimerize with retinoid X receptor-α (RXR) to exert their transcriptional activation (Armoni et al., 2003). On the other hand, previous studies regarding the transcriptional regulation of the GLUT4 gene by PG-J2 are conflicting. Treatment of explanted aortas with PG-J2 caused an increase in GLUT4 expression (Atkins et al., 2005), while in primary rat adipocytes stimulation with this endogenous agonist repressed GLUT4 promoter activity (Armoni et al., 2003).

Muscle contraction and chronic contractile activity of skeletal muscle cells have also been reported to stimulate GLUT4 gene transcription (Slentz et al., 1992; MacLean et al., 2002; Silva et al., 2005; Lima et al., 2009). In the present study, we have used electrical pulse stimulation in differentiated C2C12 myotubes to mimic the effects of exercise *in vitro.* Interestingly, all the deletion constructs containing the HIF-1 motif showed a significant increase in the transcriptional activity in response to electrical pulse stimulation. This result is in agreement with previous data indicating that HIF-1 binding factor participates in contraction-induced GLUT4 transcriptional activity (Silva et al., 2005; Lima et al., 2009). Furthermore, this supports the notion that GLUT4 expression is induced under hypoxic conditions (Royer et al., 2000) and provides a new evidence for the important role of HIF-1 as an activator of GLUT4 transcription when the partial tension of oxygen falls in muscle fibers during exercise (Wagner, 2001).

In summary, our results indicate that we have cloned the first functional GLUT4 promoter in a non-mammalian species. The Fugu GLUT4 promoter presents most of the binding sites described as important for the transcriptional regulation of the mammalian GLUT4 gene. Moreover, being consistent with data available in mammals, the Fugu GLUT4 promoter is regulated by insulin, PPARγ and contractile activity. Although most of the mechanisms involved in the transcriptional regulation by these stimuli remain unclear, this study contributes to the understanding of the complex regulation of the GLUT4 transcriptional machinery.

Acknowledgements

We are deeply indebted to Dr. Shugo Watabe for the gift of the Fugu muscle sample and Dr. Rafael Salto for the gift of the rat GLUT4 promoter. This study was funded by grants AGL2009-07006 and CSD2007-0002 to J.V.P. Rubén Marín-Juez was supported by an FPI fellowship from the Spanish government.

Chapter II

GLUT2:
Physiological role during early development in teleost fish

"It is those who know little, and not those who know much, who so positively assert that this or that problem will never be solved by science"

Charles Darwin

1. Introduction

The cellular uptake of glucose represents the main energy source for many organisms and its entry into cells is facilitated by the family of passive diffusion glucose transporters (GLUTs). The 14 different members of the GLUT family are integral membrane proteins that contain 12 membrane-spanning helices with both the amino and carboxyl termini exposed to the cytosol (Wood and Trayhurn, 2003; Augustin, 2010). Each glucose transporter isoform plays a specific role in glucose metabolism as determined by its pattern of tissue expression, substrate specificity, transport kinetics, and regulated expression under different physiological conditions (Uldry and Thorens, 2004). Interestingly, the intestine, pancreas, kidney and liver, which all play key roles in carbohydrate metabolism, express the facilitative glucose transporter GLUT2 (Thorens et al., 1988; Fukumoto et al., 1989; Bell et al., 1990; Thorens et al., 1990).

Functionally, GLUT2 is a low-affinity transporter for glucose, fructose, mannose, and galactose, but it is also a high–affinity transporter for glucosamine (Uldry et al., 2002). As a class I glucose transporter, GLUT2 is predicted to have the consensus structure characteristic of GLUTs belonging to this family with the only exception that the QLS motif in helix 7 is not present (Burant et al., 1991; Baldwin, 1993). This motif is known to confer substrate specificity and its absence in GLUT2 could explain the high affinity of this transporter to D-glucose, D-fructose and glucosamine (Burant et al., 1991; Baldwin, 1993; Uldry et al., 2002).

In mammals, GLUT2 is expressed at a very high level in pancreatic β-cells, being more abundant in the microvilli than in the basolateral membrane (Orci et al., 1989). GLUT2 is also expressed in the basolateral and apical membranes of intestinal and kidney epithelial cells (Thorens et al., 1990; Thorens, 1992; Kellett and Helliwell, 2000; Marks et al., 2003; Wright et al., 2003) as well as in the sinusoidal plasma membrane and in endosomal fractions of hepatocytes (Thorens et al., 1990; Thorens, 1992; Eisenberg et al., 2005). From a physiological point of view, the expression pattern of

GLUT2, together with the ability of GLUT2 to transport different types of hexoses at a wide range of concentrations, ensures fast equilibration of glucose between the extracellular space and the cell cytosol, displaying bidirectional fluxes in and out of the cells (Leturque et al., 2005; Thorens and Mueckler, 2010). This mechanism contributes to a number of different processes, such as intestinal and renal absorption of glucose, the stimulation of insulin secretion by glucose in β-pancreatic cells, the entry and output of glucose by the liver and the glucosensing capability of specific brain regions involved in the regulation of glucose metabolism and food intake (Burcelin et al., 2000; Guillam et al., 2000; Uldry and Thorens, 2004; Leturque et al., 2005; Marty et al., 2007; Eny et al., 2008).

Amongst non-mammalian vertebrates, GLUT2 has been to date identified and studied at the molecular level in avian and fish species (Wang et al., 1994; Krasnov et al., 2001; Hall et al., 2006; Castillo et al., 2009; Terova et al., 2009). Particularly, studies in teleost fish have shown that GLUT2 is expressed in the pancreas, liver and hypothalamus in rainbow trout and Atlantic cod and in brain, heart, liver, kidney, muscle and intestine in sea bass (Panserat et al., 2001; Hall et al., 2006; Polakof et al., 2007; Terova et al., 2009) while in adult zebrafish GLUT2 expression was found mainly in testis, brain, skin, kidney, intestine, liver and muscle (Castillo et al., 2009). Furthermore, Castillo et al. showed that the sugar transport properties of zebrafish GLUT2 are similar to what has been observed in mammals (Castillo et al., 2009). These data confirmed the high degree of functional conservation between fish GLUT2 and its mammalian homolog.

Regarding the physiological role of GLUT2, little is known to date in fish. It has been shown that in fish, the expression of GLUT2 in the pancreas and in the hindbrain is regulated by hormonal and metabolic signals (Polakof et al., 2007) whereas GLUT2 mRNA expression levels in the liver are not affected in fasting/re-feeding conditions (Panserat et al., 2001; Hall et al., 2006). However, mRNA levels of fish GLUT2 in the liver are up-regulated in acute and long-term hypoxic conditions (Terova et al., 2009).

Furthermore, recent studies have evidenced that GLUT2 expression in zebrafish is also under nutritional regulation in the intestine (Castillo et al., 2009).

In the present study, we have used the developing zebrafish embryo model to examine *in vivo* the physiological role of GLUT2. The ease of genetic manipulation, rapid development and optical transparency of the zebrafish embryo provides a unique opportunity to unravel the mechanisms following the perturbation of glucose homeostasis associated with loss of function of this transporter. Our findings indicate that the abrogation of zebrafish GLUT2 *in vivo* results in severe abnormalities in the development of the brain and particularly in the neural progenitor cells. These alterations are associated with impaired glucose uptake and a significant increase in cell apoptosis in the morphant embryos. In addition, knockdown of GLUT2, coinciding with the observed tissue distribution of this transporter, leads to changes on the patterning of endoderm-derived tissues at early developmental stages.

2. Materials and methods

Zebrafish maintenance

Wild-type zebrafish of the AB/TL and Casper strains were maintained and staged as described previously (Kimmel et al., 1995; Westerfield, 2000) in compliance with Animal Welfare legislation.

Imaging

Embryos were examined with a Leica MZ16 FA fluorescence stereomicroscope and images were acquired with a DFC420C camera (Leica) and Leica Application Suite 3.8 (LAS) Microscope Software. Pictures were analyzed using ImageJ 1.45 software (National Institutes of Health, NIH) and the fluorescent signal was measured according

to Tucker and Lardelli (Tucker and Lardelli, 2007). TUNEL-positive cells were analyzed and quantified using Icy 1.2.4.1 bio–imagining software (www.bioimageanalysis.org).

In situ hybridization and immunohistochemistry

In situ hybridization was performed as previously described (Thisse and Thisse, 2008). Antisense probes were generated for zebrafish GLUT2 (Castillo et al., 2009) and for transferrin, elastase b pre-proinsulin ATOH1b, ATOH1c, PTF1a and NEUROD that were amplified by PCR and subcloned into pGEM-T Easy vector (Promega). GLUT2 was linearized with SpeI and ATOH1b, ATOH1c, PTF1a and NEUROD were linearized with SalI and used as template for the generation of riboprobes using the DIG and Fluorescein labeling kits (Roche Applied Science).

For whole-mount immunostaining, zebrafish embryos were fixed in 4%PFA and washed with PBS (pH 7.4) containing 1% dimethyl sulfoxide (DMSO, Merck, Germany) and 0.3% TritonX-100 (Sigma-Aldrich) (PBS-DTx) at room temperature. Embryos at 24 and 48 hpf were digested with collagenase type IA (Sigma-Aldrich) diluted in PBS-DTx (1mg/ml) at 37ºC for 10 and 20 min, respectively. Next, after 2 to 5 hours of incubation in blocking solution (PBS-DTx with 5% sheep serum) the specimens were incubated with the anti-acetylated tubulin antibody (Sigma-Aldrich) diluted (1:200) in blocking solution during 16 hours at 4°C under slow stirring (30-50 rpm). Embryos were then washed thoroughly with PBS-DTx and incubated with the secondary antibody, goat anti-mouse Alexa-conjugated 488 diluted (1:500) in blocking solution for 12–24 hours at 4°C. After extensive washing with PBS-DTx (pH 7.4), the specimens were stored in PBS.

Morpholino design and injections

To knock down zebrafish GLUT2 expression, we designed antisense morpholinos targeting the translational start site (5′-ACTGCTTCTCCATTTTGCATGAAGT-3′) and the splice acceptor site of exon 6 (5′-ATGACCTGCAGACAACAAGGACACC-3′).

Morpholinos were reconstituted in RNAse-free water according to manufacturer's instructions (Gene Tools LLC, Philomath, OR). Morpholinos targeting the translational start site (ATG MO) and the splice acceptor site (splice MO) were titrated at doses of 2.2–8.4 ng into single-cell embryos and the lowest effective dose was determined (3.1 ng) and used for all subsequent experiments. A standard control morpholino (5′-CCTCTTACCTCAGTTACAATTTATA-3′) (Gene Tools LLC) was used as negative control. Capped mRNAs were synthesized from rat *Glut2* and zebrafish GLUT2 full-length cDNAs cloned into pcDNA3 and pBK-CMV vectors respectively, using mMessage mMachine kit (Ambion). 250 pg/embryo of rat GLUT2 and 150 pg/embryo of zebrafish GLUT2 mRNAs were co-injected with 3.1 ng/embryo of the ATG MO and the splice MO, respectively.

In vivo glucose uptake assay

Control and ATG morphants and rescued embryos were injected at 24 hpf in the yolk sac with 2.5 mg/ml 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG), a fluorescent glucose analog (Life Technologies), and incubated at 28.5 ºC for 60 min. At the termination of the incubation period, 7 embryos per condition were anesthetized with 3-aminobenzoic acid ethyl ester methanesulfonate (Sigma-Aldrich) and analyzed under a fluorescence stereomicroscope. The fluorescent signal was measured as described above. To visualize the transport of glucose, the embryos were embedded in 1% methylcellulose.

Detection of apoptotic cell death

Cell death was detected *in vivo* using the vital dye acridine orange (acridinium chloride hemizinc chloride; Sigma). Embryos were dechorionated and incubated with acridine orange (5 µg/ml) for 30 min at 28.5 ºC in the dark. At least 7 embryos per condition were washed three times with egg water for 5 min and immediately visualized with a fluorescence stereomicroscope and the fluorescent signal was measured as

described above. Apoptosis in zebrafish whole mount embryos fixed with 4% PFA was detected by TUNEL using the In Situ Cell Death Detection Kit (Roche) following the manufacturer's protocol. For the quantification of TUNEL positive cells at least 3 embryos per condition were analyzed as described above.

PCR analysis

Total RNA was reverse transcribed with SuperScript III RNase H^- Reverse Transcriptase (RT; Invitrogen) and conventional PCR was performed with recombinant Taq DNA Polymerase (Invitrogen) and specific primers for zebrafish GLUT2 (For. 5'-CTGGCTATTGTCATTGGCATCC-3'; Rev. 5'-TGTCCTTAGAGGTGTCATAATCTCCC-3'). PCR products were resolved in a 1.2 % agarose gel and stained with SYBR® Safe DNA Gel Stain (Invitrogen) for visualization.

Microarray design

Access to the custom microarray platform used in this study was kindly provided by Prof. Herman P. Spaink (University of Leiden, The Netherlands). The microarray slides were custom-designed by Agilent Technologies. The slides contained in total 43,371 probes of a 60-oligonucleotide length. Of these probes, a total of 21,496 probes were identical to the probes present on the Agilent probe set that is commercially available under catalog no. 013223_D. The additional probes were designed using the eArray software from Agilent Technologies (earray.chem.agilent.com/earray). Settings used were based on the following settings: base composition methodology, best probe methodology, and design with 3 bias. The Agilent *Danio rerio* transcriptome was used as a reference database. The microarray design has been submitted to the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo) under accession no. GPL13390.

Microarray analysis

Control and ATG morphant embryos were harvested at 72 hpf and RNA samples were obtained from pools of 20 embryos per condition and three pooled biological replicates of control and ATG morphants were analyzed. RNA was amplified and labeled with Cy3 dye using single color Low Input Quick Amp Labeling kit (Agilent Technologies) following the manufacturer's indications using 200 ng of RNA in each reaction. Next, 825 ng of labeled cRNA were hybridized to the arrays. Overnight hybridization (17 hrs, 65ºC and 10 rpm rotation) was performed in a Microrarray Hybridization Oven (Agilent Technologies). After hybridization, microrarrays were washed with Gene Expression Wash Buffers 1 and 2 (Agilent Technologies) and scanned using the High-Resolution C Scanner (Agilent Technologies).

Feature Extraction Software 10.7.3 (Agilent Technologies) was used for spot to grid alignment, feature extraction and quantification. Processed data were subsequently imported into GeneSpring GX 11.5 (Agilent Technologies). Significance cut-offs for the ratios of control vs ATG morpholino were set at 1 and 1.5-fold change at $p<0.01$ and 0.05 (sample t-test), respectively, for differentially expressed genes (DEGs). For the DEGs, gene IDs were converted to human ENSEMBL gene IDs using g:orth function from G:profiler (http://biit.cs.ut.ee/gprofiler), taking advantage of the more complete gene ontology (GO) annotations of the human genes and improving, in this way, the subsequent analysis of the functional categories.

GO enrichment analysis was performed using Database for Annotation, Visualization and Integrated Discovery (DAVID) software tools (http://david.abcc.ncifcrf.gov) (Huang et al., 2008), and the resulting categories were considered significant at $p\leq0.05$.

Statistical analysis

Results are expressed as mean ± SE. Statistical differences were analyzed by Kruskal-Wallis and Mann-Whitney non-parametric tests and considered to be significant at $p \leq 0.05$.

3. Results

Expression pattern of zebrafish GLUT2

To determine the expression pattern of zebrafish GLUT2, we synthesized an antisense RNA probe and performed ISH. At 24 hpf, GLUT2 expression was localized in the telencephalon, eyes hindbrain and pronephric duct (Fig. 1A, B). By 48 hpf, we detected strong expression of GLUT2 in the head and in the pronephric duct (Fig. 1C). At 72 and 120 hpf, GLUT2 appeared expressed in the liver, pronephric tubules, anterior intestine, endocrine pancreas and hindbrain (Fig. 1D-J). In addition, we confirmed that the observed expression in the pancreas region corresponded to endocrine pancreas by performing a double ISH using a RNA probe for pre-proinsulin as specific marker for this tissue (Fig. 1J).

Knockdown of zebrafish GLUT2

To study the function of GLUT2 during the early developmental stages in zebrafish, we designed two antisense MO, one to inhibit translation at the start methionine (ATG MO) and a second one designed to interfere with splicing between exons 5 and 6 (Splice MO).

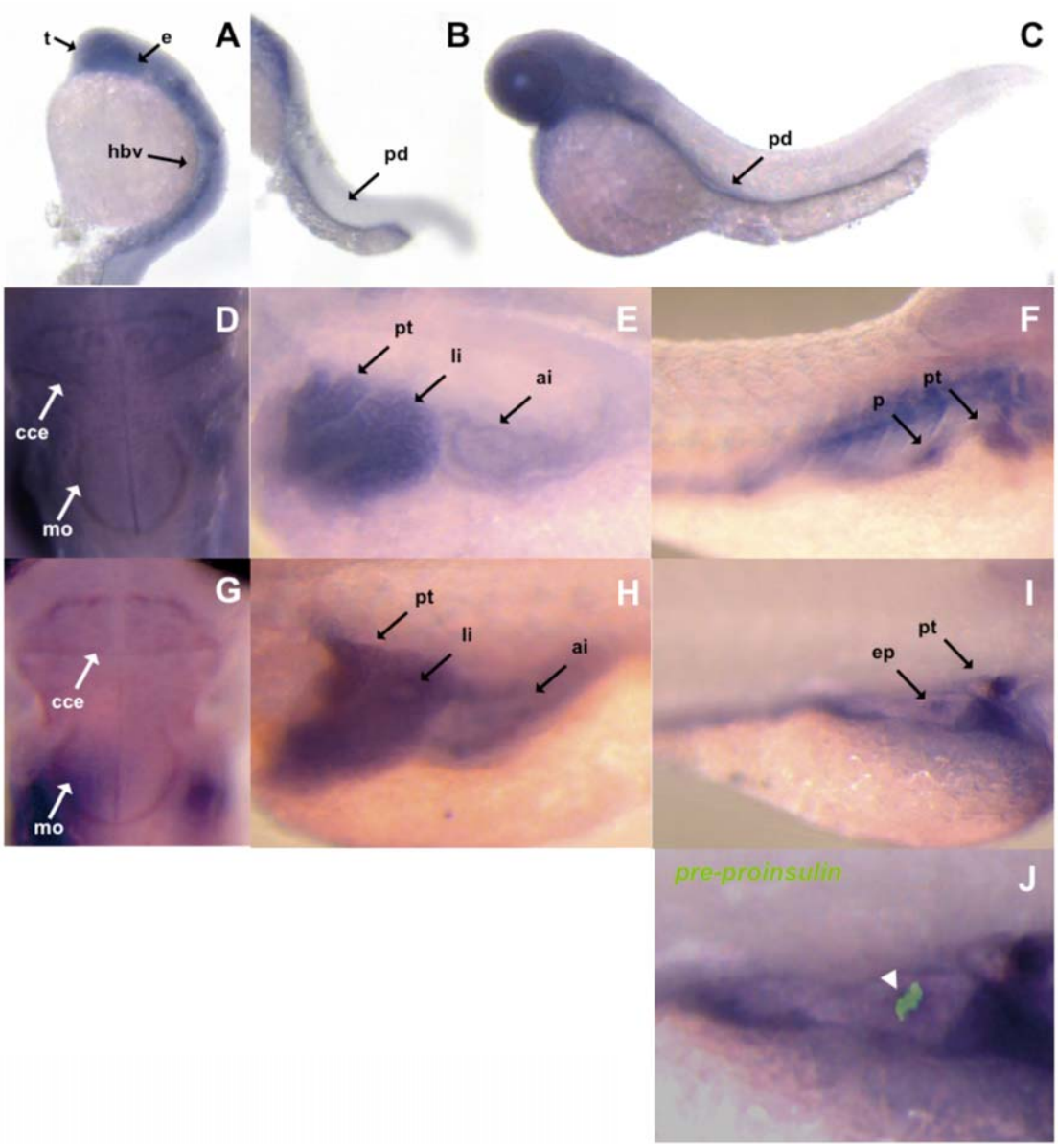


Figure 1. Localization of the expression of GLUT2 in zebrafish. Whole mount *in situ* hybridization showing expression of zebrafish GLUT2 mRNA at 24 hours post fertilization (hpf) (A, B), 48hpf (C), 72hpf (D-F) and 120hpf (G-J). Left (A-C, E,H), right lateral views (F, I, J) and dorsal views of the hindbrain region (D, G) are shown. To confirm GLUT2 expression in endocrine pancreas, double *in situ* hybridization showing pre-proinsulin expression was performed (J). Anterior intestine (ai), corpus cerebelli (cce), endocrine pancreas (ep), eye (e), hindbrain ventricle (hbv), liver (li), medulla oblongata (mo), pronephric duct (pd), pronephric tubule (pt), telencephalon (t). White arrowhead indicates endocrine pancreas as shown by the presence of pre-proinsulin signal.

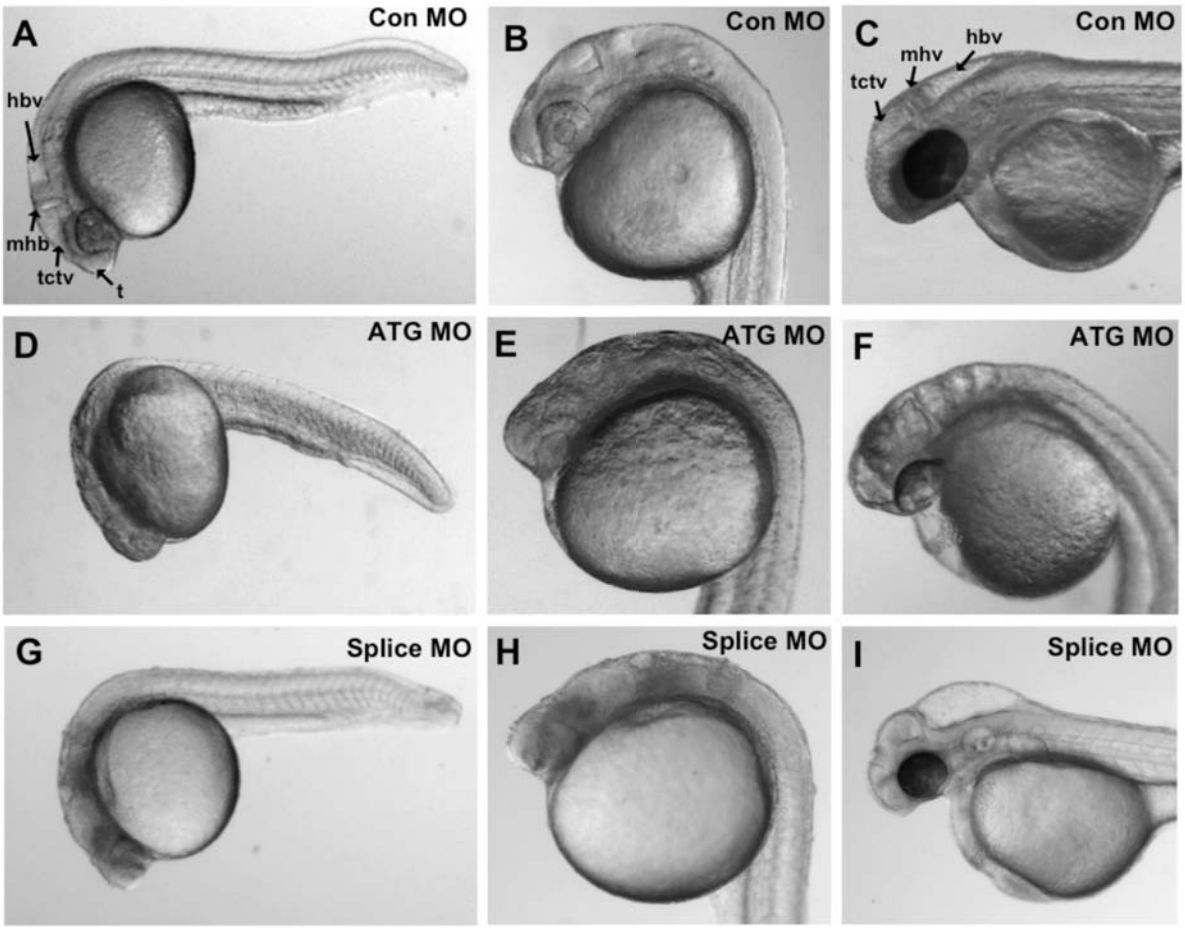


Figure 2. Morphant phenotype caused by abrogation of GLUT2. Phenotype of embryos injected with a control morpholino (Con MO) at 24 hours post fertilization (hpf) (A, B) and at 48 hpf (C), a morpholino targeting the translational start site (ATG MO) at 24 hpf (D, E) and at 48 hpf (F) and a morpholino targeting the splice acceptor between exons 5 and 6 (Splice MO) at 24 hpf (G, H) and at 48 hpf (I). Hindbrain ventricle (hbv), midbrain/hinbrain boundary (mbh), tectal ventricle (tctv), telencephalon (t).

Embryos injected with the ATG MO showed a severe delay in the development of the embryo mainly in the brain area. Morphants at 24 hpf showed less developed eyes and a dense mass was observed in the brain area instead of the hindbrain ventricle, causing the loss of the midbrain/hindbrain structures (Fig. 2D, E). Embryos injected with the Splice MO appeared to be a phenocopy of the ATG morphants confirming the specificity of the morpholino (Fig. 2G, H). By 48 hpf, morphant embryos displayed a

defective formation and enlargement of the hindbrain ventricle associated with anterior displacement of the telencephalon (Fig. 2F, I). MO injected embryos did not survive past 96-120hpf.

The incidence of the morphant phenotype was 95.9 ± 1.4% in embryos injected with the ATG MO and 93.4 ± 1.2% in embryos injected with the Splice MO. Furthermore, analysis of GLUT2 transcripts from MO treated embryos by ISH revealed a significant reduction in GLUT2 expression following injection of the ATG MO (Fig. 3) and the appearance of an aberrant splice product, confirmed by nucleotide sequence analysis, in embryos injected with the splice site MO (Fig. 4).

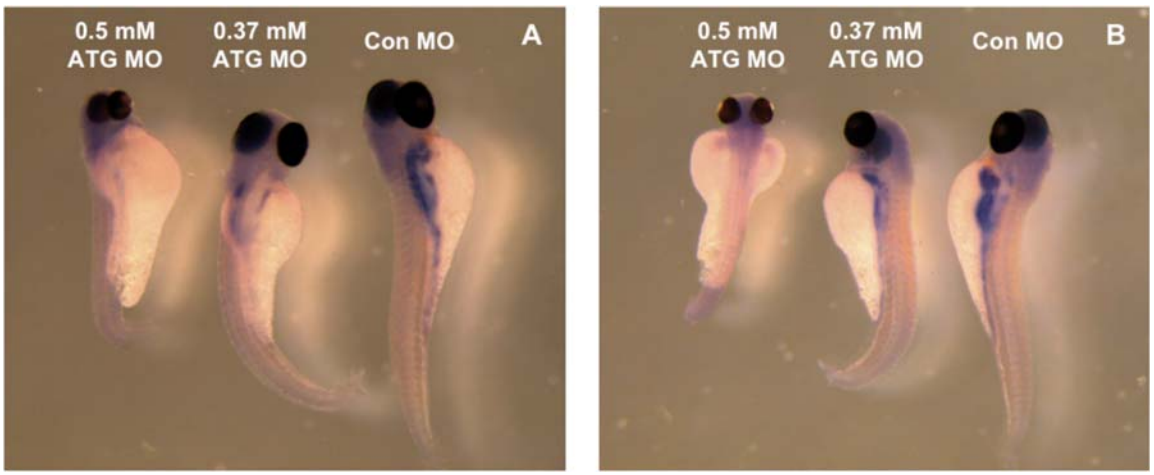


Figure 3. *In situ* hybridization of GLUT2 in morphant embryos. GLUT2 expression appears reduced in embryos injected with ATG MO in a dose-dependent manner. Right (A) and left (B) views of embryos at 72 hours post fertilization injected with control morpholino (Con MO) and ATG morpholino (ATG MO) at a concentration of 0.37 and 0.5 mM.

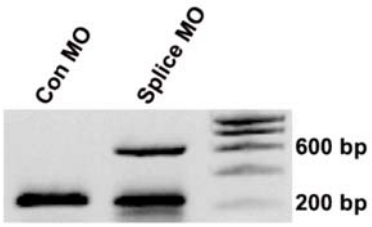


Figure 4. Disruption of GLUT2 mRNA splicing by a splice morpholino. RT-PCR analysis of embryos at 24 hours post fertilization injected with control morpholino (Con MO) or the splice morpholino (Splice MO) using primers flanking the GLUT2 targeted exon designed to generate differentially sized transcripts. Embryos injected with the Splice MO reveal the appearance of a larger transcript (600 bp).

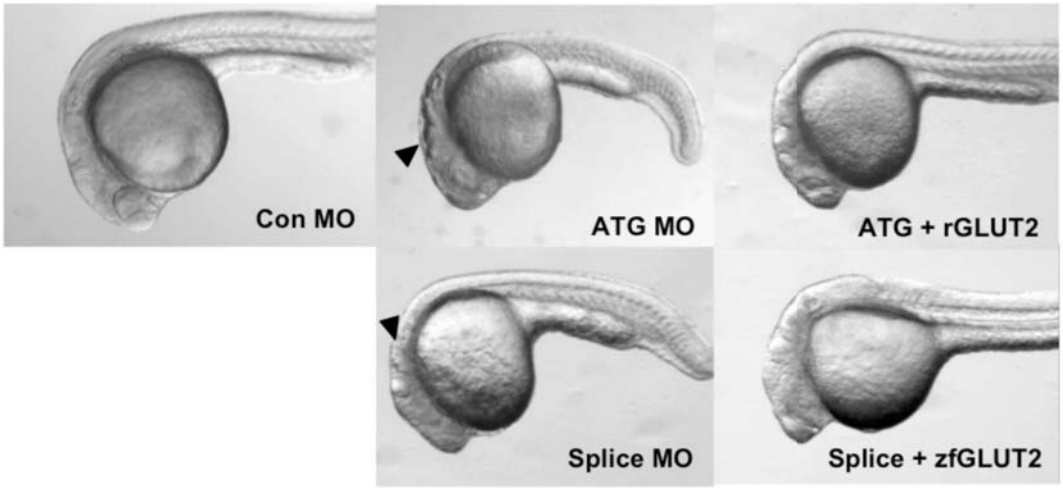


Figure 5. Rescue of the morphant phenotype with full-length zebrafish GLUT2 and rat GLUT2 mRNA. Rescue of overall morphant phenotype at 24 hours post fertilization by co-injecting ATG MO-injected embryos with rat GLUT2 mRNA (rGLUT2) and Splice MO- injected embryos with zebrafish GLUT2 (zfGLUT2). Morphant embryos presented deficient hindbrain formation (arrowhead).

To further demonstrate the specificity of the GLUT2 morphant phenotype, we performed rescue experiments by co-injecting rat GLUT2 and zebrafish GLUT2 mRNA together with the ATG and Splice MO, respectively. This analysis revealed that both zebrafish GLUT2 and rat GLUT2 mRNAs were able to rescue the morphant phenotype after 24 and 48 hpf, showing a normal brain and body development (Fig. 5, 6). The

rescue achieved a maximum of 20% of wild type phenotype embryos in ATG morphants co-injected with rat GLUT2 mRNA and 64% phenotypic rescue in Splice MO co-injected with zebrafish GLUT2 mRNA (Fig. 6), representing an increase of 5.6 and 7.7 fold in the percentage of wild type embryos.

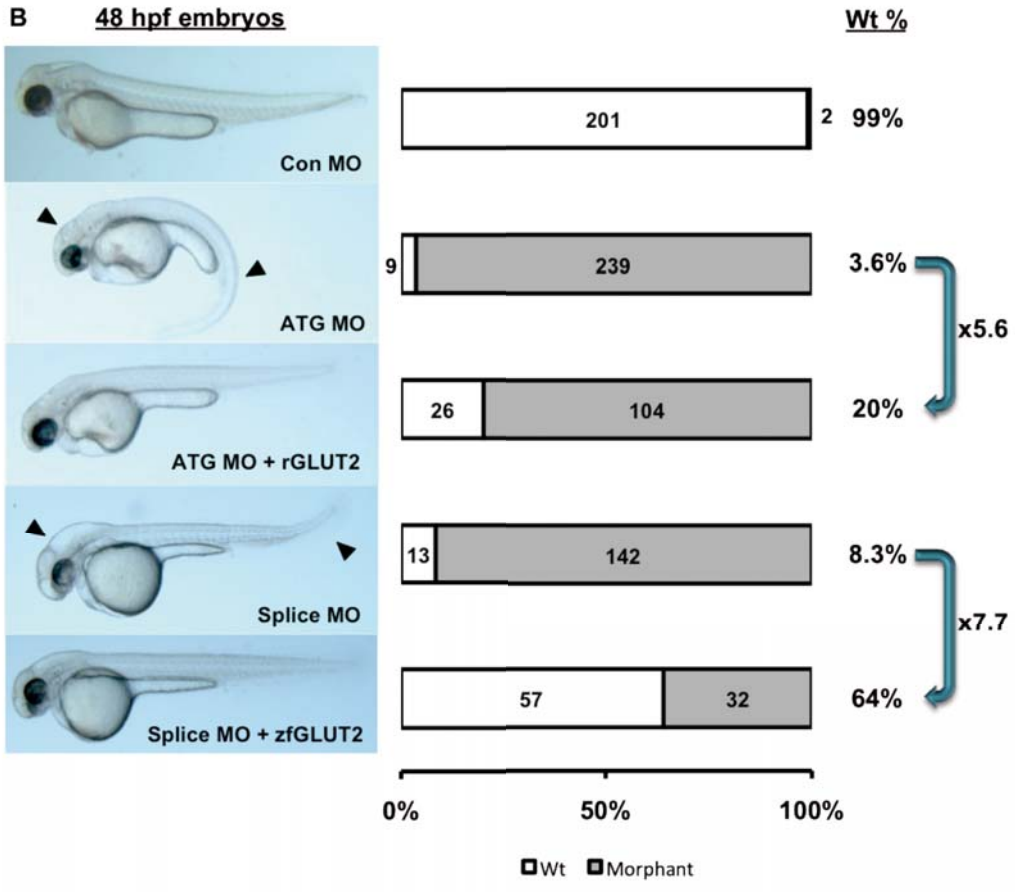


Figure 6. Rescue of the morphant phenotype with full length zebrafish GLUT2 and rat GLUT2 mRNA. Rescue of overall morphant phenotype at 48 hours post fertilization by co-injecting full length ATG injected embryos with rat GLUT2 mRNA (rGLUT2) and Splice injected embryos with zebrafish GLUT2 (zfGLUT2). Morphant embryos presented deficient hindbrain formation and curved back (arrowhead). Percentages indicated correspond to percentage of wild type phenotype embryos (Wt) present in each case.

To characterize the effects of GLUT2 abrogation on the central nervous system in morphant zebrafish embryos we performed immunohistochemical analysis using an antibody against neuron-specific acetylated tubulin. By 24 hpf, a simple axon scaffold had formed in the embryonic zebrafish brain, consisting of two bilaterally symmetrical longitudinal tracts connected by commissures, providing a template for subsequent development (Fig. 7A). In contrast, the neuronal architecture of GLUT2 morphants was clearly altered, revealing thinner, poorly-fasciculated longitudinal tracts (Fig. 7B). Morphant embryos co-injected with rat GLUT2 mRNA recovered the brain structure similar to control injected embryos (Fig. 7C). At 48 hpf, control and ATG morphant embryos presented no significant differences in the axonal structure (Fig. 7D, E).

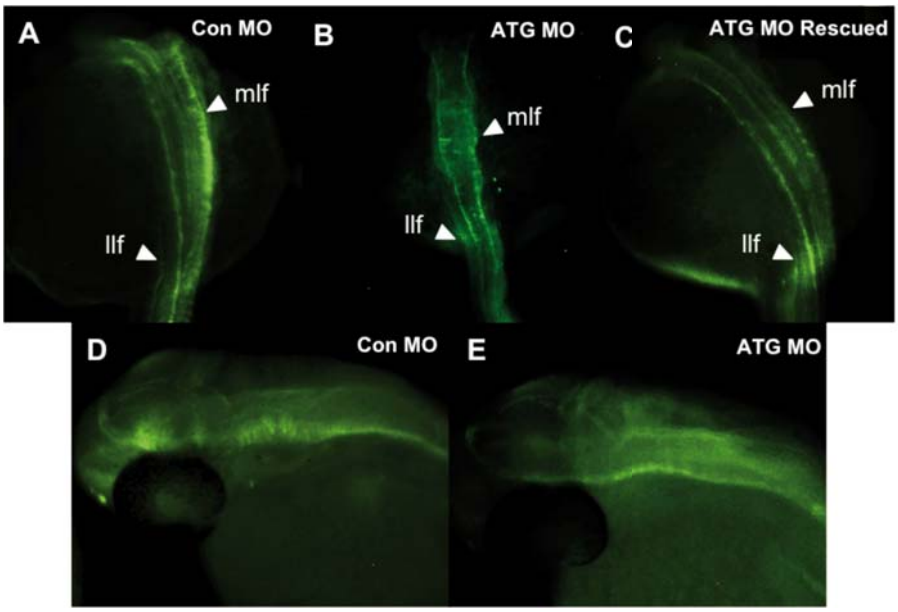


Figure 7. Morphological characterization of longitudinal axon tracts in the hindbrain of GLUT2 deficient fish. To study the consequences of GLUT2 abrogation in the hindbrain structure, embryos injected with control morpholino (Con MO) (A), ATG morpholino (ATG MO) (B) or embryos rescued with rat GLUT2 mRNA (ATG MO Rescued) (C) at 24 hours post fertilization (hpf) were immunostained using an antibody against acetylated tubulin. At this stage, morphant embryos showed disorganized axon tracts. Rescued embryos showed a hindbrain structure similar to control injected embryos. By 48 hpf, embryos injected with Con MO (D) and ATG MO (E) presented no differences. Lateral longitudinal fascicles (llf); medial longitudinal fascicles (mlf).

Knockdown of GLUT2 influences brain development in zebrafish by affecting the expression of markers for cerebellar progenitor cells

To assess the effects of GLUT2 abrogation in the hindbrain region we performed ISH for various proneural genes: PTF1Aa, ATOH1b, ATOH1c and NEUROD (Fig. 8). The probe for PTF1A marks progenitor cells of GABAergic neurons in the ventricular zone (VZ) (Kani et al., 2010). ATOHb and ATOHc probes label progenitor cells of glutamatergic neurons while NEUROD appears expressed in immature and mature granule cells (Kani et al., 2010). Embryos injected with ATG MO lacked expression of PTF1a at 24 hpf and at 48 hpf morphant embryos showed an expression pattern similar to that in control embryos at 24 hpf (Fig. 8A-D). Proneural gene ATOH1b, although appearing expressed in both control and ATG morphants, denoted severe alterations in the hindbrain structure at 24 and 48 hpf, with no apparent effects at 72 hpf (Fig. 8E-J). NEUROD expression in ATG morphants appeared mildly affected at 24 hpf, showing loss of expression in the anterodorsal/anteroventral line facial placode ganglia (ad/av/f) (Fig. 8L). At 48 and 72 hpf, embryos showed no significant alteration in NEUROD expression (Fig. 8M-P). ATOH1c expression was not altered by 72 hpf (Fig. 8Q, R).

Defective glucose uptake in GLUT2-deficient embryos

In order to evaluate the functional impact of inhibiting GLUT2 expression, we performed glucose uptake experiments using the fluorescently labeled glucose analog, 2-NBDG. Quantitative analysis of the fluorescent signal revealed a significant decrease in 2-NDBG uptake in the head and body in ATG morphant embryos relative to controls, keeping most of the glucose inside the yolk (Fig. 9A, B). In contrast, ATG morphants rescued with the rat GLUT2 mRNA appeared to recover glucose uptake showing similar levels relative to control embryos in body and yolk and a slight increase in the body (Fig. 9A, B).

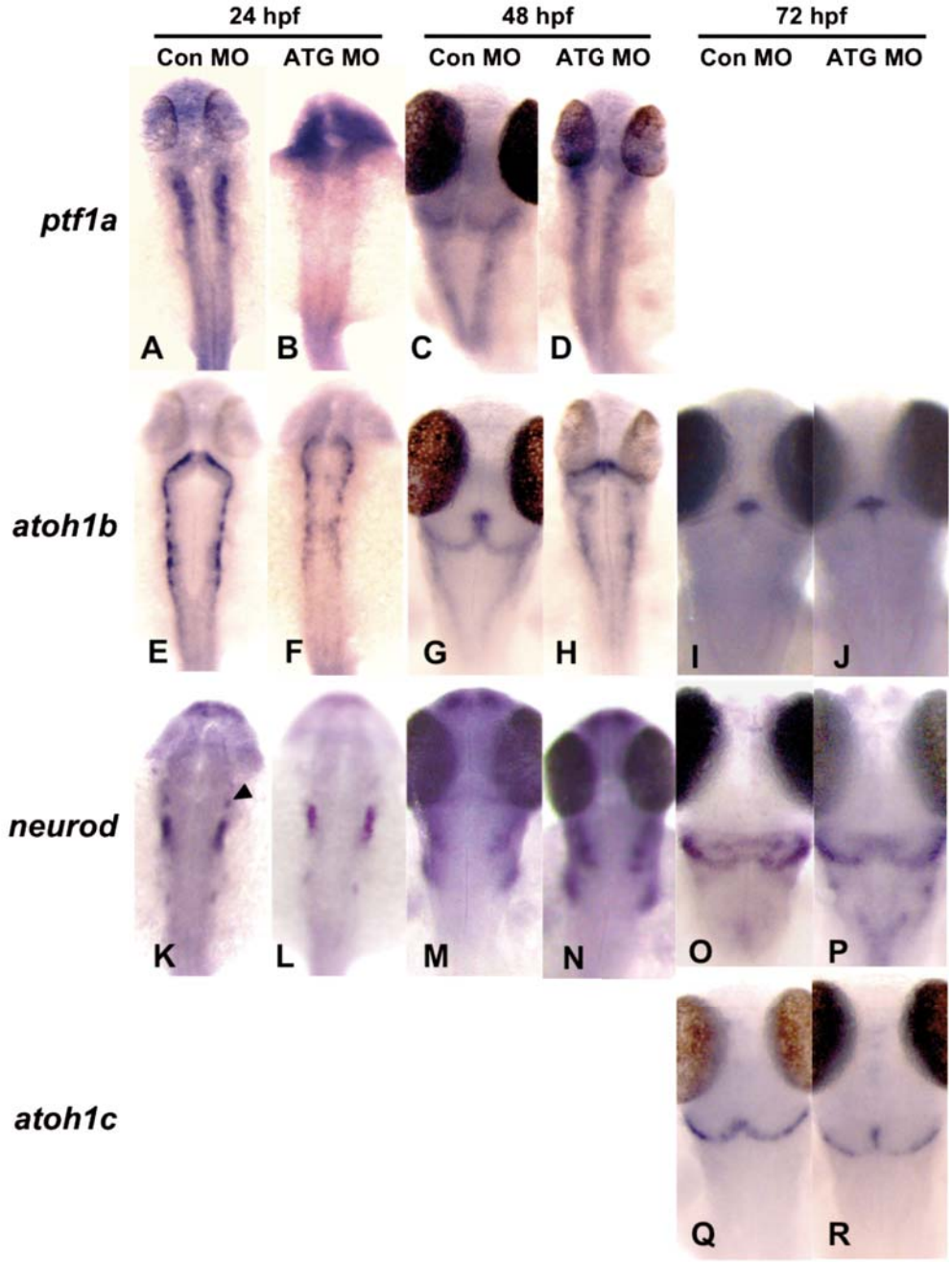
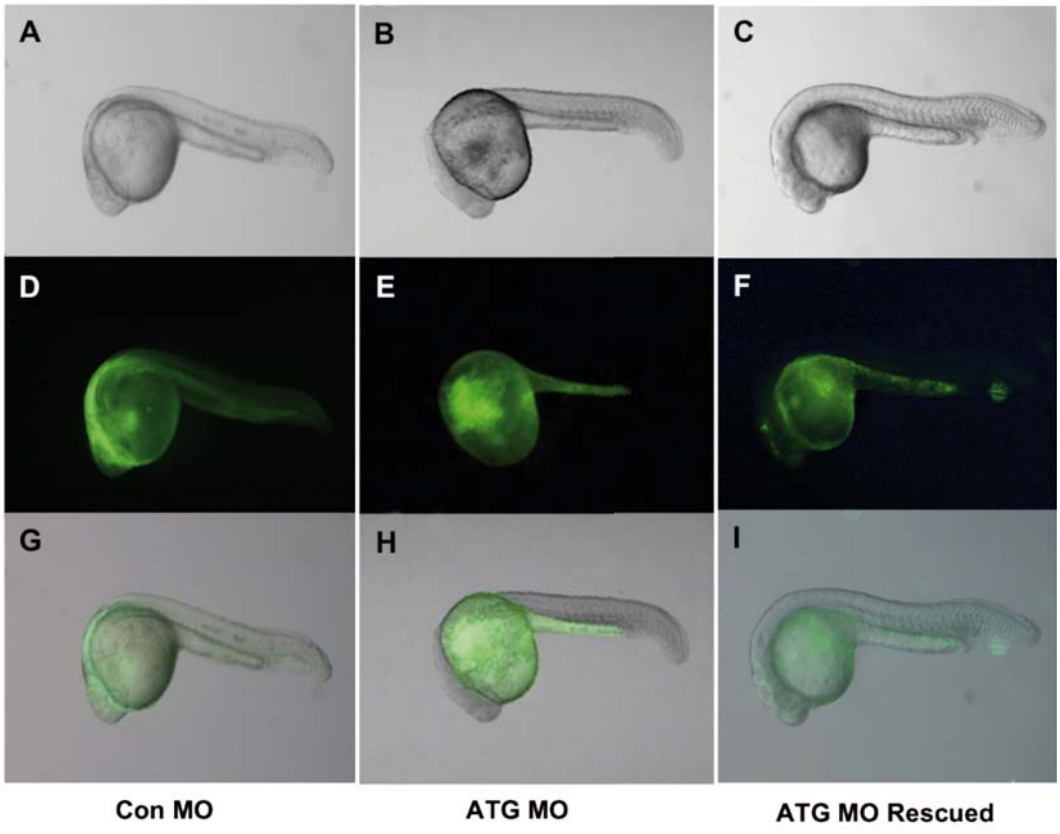


Figure 8. Expression of proneural genes during cerebellar development in GLUT2 morphants. Expression of PTF1a (A-D), ATOH1b (E-J), NEUROD (K-P) and ATOH1c (Q,R) in control injected embryos at 24 hours post fertilization (hpf) (A, E, K), 48 hpf (C, G, M) and 72 hpf (I, O, Q); and in ATG morphants at 24 hpf (B, F, L), 48 hpf (D, H, N) and 72 hpf (J, P, R). Anterodorsal/anteroventral line facial placode ganglia (arrowhead).

A


A
B
C
D
E
F
G
H
I
Con MO
ATG MO
ATG MO Rescued

B

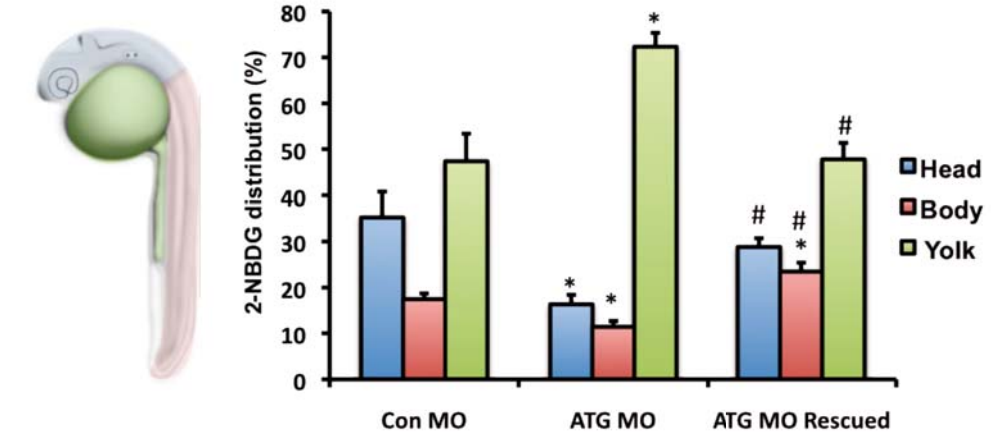

2-NBDG distribution (%)
80
70
60
50
40
30
20
10
0
Head
Body
Yolk
Con MO
ATG MO
ATG MO Rescued

Figure 9. Abrogation of GLUT2 results in inhibition of glucose uptake *in vivo*. (A) Bright field (upper line), fluorescent (middle line) and overlay (bottom line) pictures of control, ATG morphants and rescued embryos at 24 hours post fertilization. (B) Measurement of fluorescent signal in embryos injected with 2-NBDG. Control injected embryos (Con MO) (A, D, G) displayed significant amounts of fluorescent glucose throughout the embryo; in contrast, ATG morpholino injected embryos (ATG MO) (B, E, H) showed very minimal fluorescent glucose visible at 60 min after injection. Embryos injected with ATG MO + rat GLUT2 mRNA (ATG MO Rescued) recovered glucose uptake to levels similar to Con MO. * indicates significant differences compared with the Con MO injected embryos ($p<0.05$). # indicates significant differences compared with ATG MO injected embryos ($p<0.05$).

Loss of GLUT2 leads to an increase in apoptotic cell death

Based on the observed expression of GLUT2 in the hindbrain and on the consequences of GLUT2 knockdown in this brain region, we hypothesized that loss of GLUT2 in zebrafish embryos could affect the incidence of programmed cell death.

Examination of cell death at 24 hpf using the vital dye acridine orange showed a significant increase in cell death in the morphant embryos, mainly localized in the brain area (Fig. 10B, C). ATG morphants co-injected with rat GLUT2 presented similar levels of cell death to control morphants (Fig.10D). Quantitative measurement of the fluorescent signal confirmed a significant 1.6 fold-increase ($p<0.05$) in cell death in GLUT2 morphant embryos compared with control embryos, while rescued embryos showed no significant differences with control morphants (Fig. 10E).

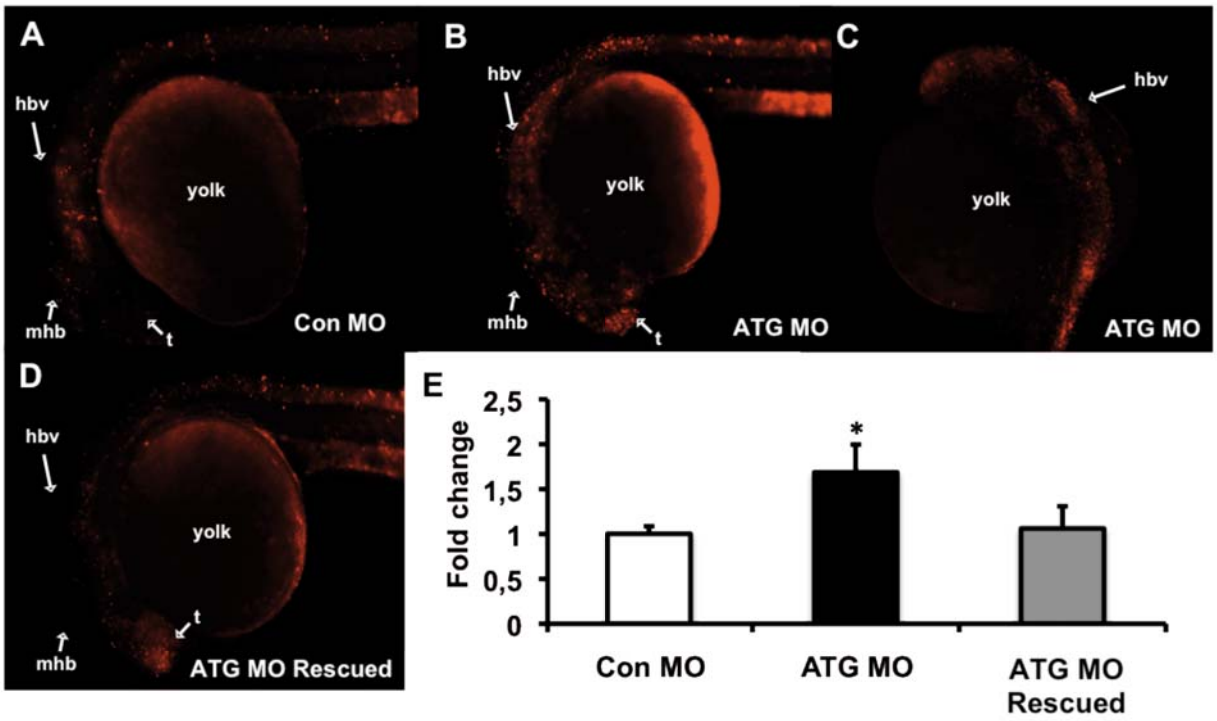


Figure 10. Abrogation of GLUT2 enhances programmed cell death. To assay for cell death, embryos injected with control morpholino (Con MO) (A), ATG morpholino (ATG MO) (B, C) and ATG morpholino + rat GLUT2 mRNA (ATG MO Rescued) (D) were stained with the vital dye acridine orange. At 24 hours post fertilization there was an overall increase in programmed cell death primarily localized in the hindbrain region. Fluorescent signal analysis confirmed a significant increase in programmed cell death in ATG morphants (E). * indicates significant differences compared with the Con MO injected embryos ($p<0.05$). Hindbrain ventricle (hbv), midbrain/hindbrain boundary (mbh), telencephalon (t).

To further confirm that the observed increase in cell death in embryos with abrogated GLUT2 expression corresponded to apoptotic cell death we performed TUNEL assay, a method that specifically labels fragmented DNA, an established marker of programmed cell death. A similar pattern of apoptosis was observed using this technique, with significantly higher apoptotic cells present in GLUT2 morphant than in control embryos at 24 hfp (Fig. 11).

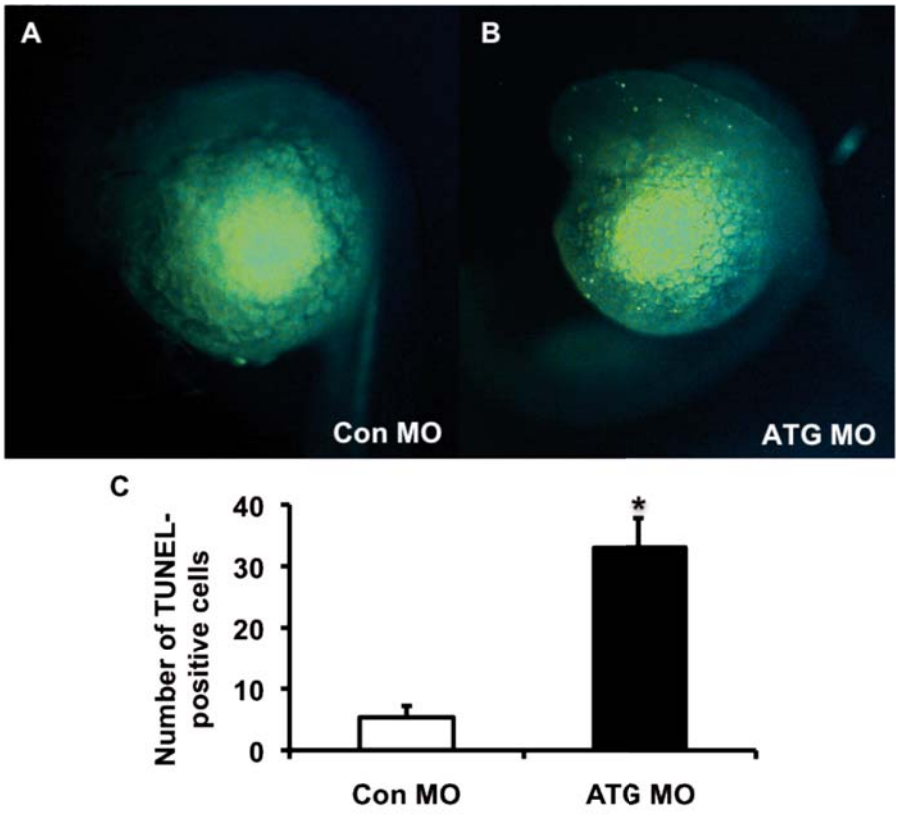


Figure 11. Embryos with GLUT2 expression blocked present a higher incidence of apoptotic cell death. To assay for apoptosis, embryos injected with control morpholino (Con MO) (A), ATG morpholino (ATG MO) (B) were detected by TUNEL assay, which preferentially stains apoptotic cells. At 24 hpf GLUT2 morphants showed a significant increase of apoptotic cells (TUNEL-positive cells) (C). * indicates significant differences compared with the Con MO injected embryos ($p<0.01$).

Abrogation of GLUT2 causes alteration in left-right asymmetry

We found that GLUT2 morphants showed altered left-right asymmetry of endoderm-derived organs that express GLUT2, such as liver and pancreas. To better characterize this effect, we performed ISH using specific probes for transferrin, elastase b and pre-proinsulin, marker genes for liver, exocrine and endocrine pancreas, respectively. Interestingly, all of them confirmed that the distribution of these organs was altered (Fig. 11).

To confirm the significance of the effect of GLUT2 abrogation on the distribution of visceral organs, we analyzed a number of GLUT2 morphant embryos (n ≥ 15) by ISH using specific probes for transferrin, elastase b and pre-proinsulin (Fig. 12). We observed that GLUT2 morphants presented alterations in the left-right axis in 69% of the embryos for liver, 73% for the exocrine pancreas and 55% for the endocrine pancreas (Fig. 12).

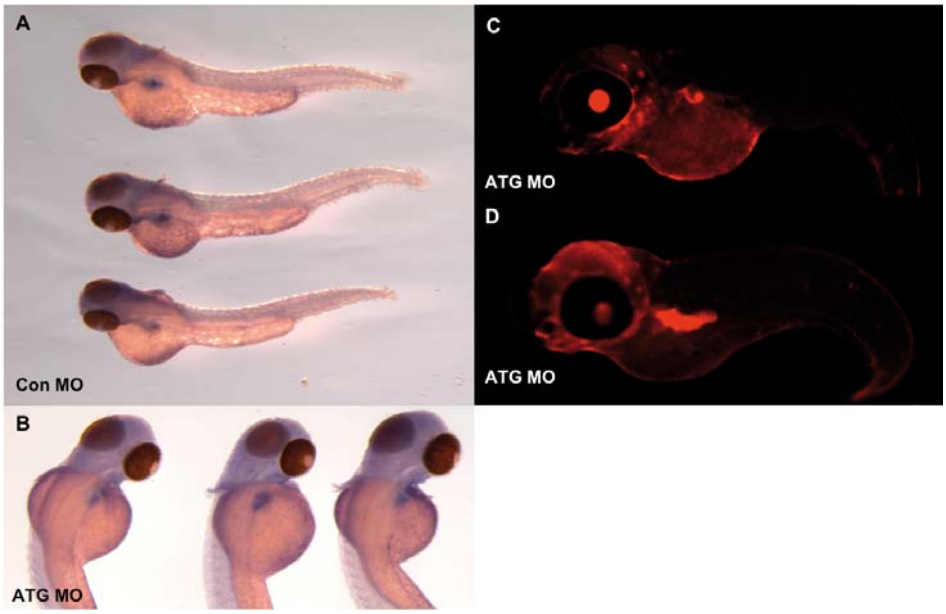


Figure 12. Expression of liver and pancreatic marker genes in GLUT2 morphants. Whole mount *in situ* hybridization showing expression of zebrafish transferrin mRNA in liver in control injected embryos (Con MO) (A) and in ATG morphant embryos (ATG MO) at 72 hours post fertilization (hpf). (B). Left side view of FISH showing expression of pre-proinsulin in endocrine pancreas (C) and elastase b in exocrine pancreas (D) in 120 hpf embryos injected with ATG morpholino.

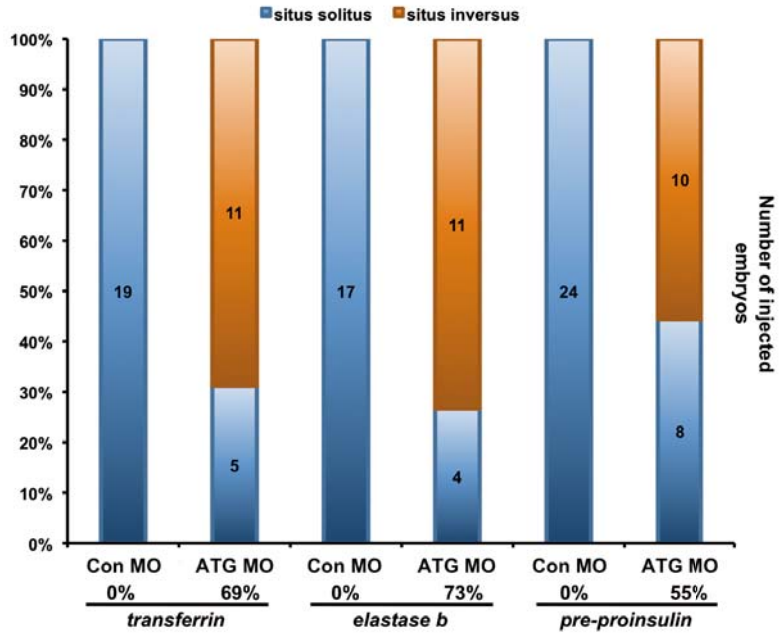


Figure 13. The left-right asymmetry of visceral organs was altered in GLUT2 morphants. Percentage of left-right asymmetry of visceral organs altered in embryos injected with ATG morpholino (ATG MO) compared with control-injected embryos (Con MO). To assess the position of the liver, the endocrine and the exocrine pancreas we performed ISH using specific probes for transferrin, elastase b and pre-proinsulin, respectively. The number of embryos showing organs *in situs solitus* or *in situs inversus* positions is indicated inside the bars.

Transcriptomic profiling of GLUT2 morphants

To study the effects of GLUT2 abrogation on gene expression in zebrafish embryos, we performed a transcriptome analysis of zebrafish embryos injected with the ATG morpholino and compared them to control embryos. We used a zebrafish oligonucleotide microarray platform previously validated and described (Stockhammer et al., 2009; Zakrzewska et al., 2010; van Soest et al., 2011) to compare the gene expression profile of ATG morphants to that of control embryos.

Microarray analysis was performed setting significance cut-offs at 1 and 1.5-fold change at $p<0.01$ and 0.05 (sample t-test), respectively. In both cases, a large number

of differentially-expressed genes (DEGs) were obtained; 1025 up-regulated and 887 down-regulated genes by applying the 1.5-fold change and $p<0.05$ cut-off settings (total n= 1912) and 726 up-regulated and 679 down-regulated genes by applying the 1-fold change and $p<0.01$ cut-off settings (total n= 1405). Next, to better characterize the annotated DEGs, we performed a GO analysis using DAVID software tools for functional classification (Table 1). Analysis of GO-Biological process revealed a significant enrichment in functional categories involved in programmed cellular death (such as anti apoptosis, apoptosis, cell death and induction to programmed cell death), muscle development (muscle organ development and muscle tissue development), neural processes (neuron projection, neurotransmitter metabolic process and visual perception), immune processes, patterning (pattern specification process and regionalization) and response to hypoxia/oxygen levels.

Analysis of the microarray results confirmed that transferrin (studied in this manuscript) and pancreatic and duodenal homeobox 1, marker genes for liver and endocrine pancreas, respectively, showed altered expression in the GLUT2 MO embryos (Table 2). Moreover, genes involved in insulin signaling and glucose metabolism appeared differentially expressed, such as Insulin receptor substrate 2, insulin-like growth factor binding protein 1, dipeptidyl-peptidase 4, ATPase, Na+/K+ transporting, alpha 1 polypeptide and calcium channel, voltage-dependent gamma subunit 2 (Table 2). In addition, GLUT2 appeared strongly down-regulated, providing further confirmation of the effectiveness of the knockdown. Furthermore, morphant embryos showed reduced expression of the left-right determination factor 2 and transforming growth factor, beta 1 (Table 2), both involved in the regulation of the expression of the Nodal pathway components, required for the normal asymmetric organization of the dorsal diencephalon and endoderm-derived tissues (Bisgrove et al., 1999; Rodaway et al., 1999; Bisgrove et al., 2000). In addition, the expression of parvalvumin, a marker gene for GABAergic neurons, was altered in morphant embryos (Table 2). Several genes involved in apoptotic cell death were also found differentially expressed, namely, programmed cell death 4, BCL2-associated agonist of cell death

and growth arrest and DNA-damage-inducible beta appeared up-regulated and apoptosis-inducing factor, mitochondrion-associated, 1, caspase 4, apoptosis-related cysteine peptidase and growth arrest and DNA-damage-inducible, gamma were down-regulated (Table 2).

Table 1. Functional annotation analysis based on GO terms

		Cutoff			
		***p*<0.05; fc >|1.5|**		***p*<0.01; fc >|1|**	
GO Term	**Name**	**Count**	**P Value**	**Count**	**P Value**
Biological process					
GO:0006916	anti-apoptosis	-	-	11	<0.01
GO:0006915	apoptosis	40	<0.01	15	<0.01
GO:0008219	cell death	47	<0.01	-	-
GO:0012502	induction of programmed cell death	-	-	15	0.04
GO:0007242	intracellular signaling cascade	70	<0.01	45	<0.01
GO:0046907	intracellular transport	-	-	26	0.04
GO:0007517	muscle organ development	18	<0.01	13	<0.01
GO:0060537	muscle tissue development	12	0.01	8	0.04
GO:0043005	neuron projection	23	0.01	-	-
GO:0042133	neurotransmitter metabolic process	5	0.01	4	0.02
GO:0044271	nitrogen compound biosynthetic process	21	0.05	17	0.01
GO:0007389	pattern specification process	20	0.01	-	-
GO:0045921	positive regulation of exocytosis	-	-	3	0.03
GO:0002684	positive regulation of immune system process	24	<0.01	-	-
GO:0032388	positive regulation of intracellular transport	5	0.03	-	-
GO:0007243	protein kinase cascade	-	-	17	<0.01
GO:0003002	regionalization	15	0.03	-	-
GO:0032844	regulation of homeostatic process	12	<0.01	10	<0.01
GO:0044057	regulation of system process	21	0.03	17	0.01
GO:0001666	response to hypoxia	14	<0.01	10	<0.01
GO:0070482	response to oxygen levels	14	0.01	10	0.01
GO:0007601	visual perception	21	<0.01	-	-
Celullar component					
GO:0030424	axon	13	0.02	9	0.04
GO:0005829	cytosol	76	<0.01	52	<0.01
GO:0005783	endoplasmic reticulum	54	0.01	37	0.01
GO:0048471	perinuclear region of cytoplasm	21	0.01	14	0.03
Mollecular function					
GO:0008289	lipid binding	27	0.05	19	0.04
GO:0000166	nucleotide binding			72	<0.01
GO:0043565	sequence-specific DNA binding	41	<0.01	28	<0.01
GO:0005212	structural constituent of eye lens	5	<0.01	-	-
GO:0003700	transcription factor activity	30	0.05	37	<0.01

The table indicates the number of genes in each list that are associated with the indicated GO term resulting from applying two different significance cutoffs; *p-value* (*p*) <0.01 and fold change (fc)>|1| and *p-value* (*p*) <0.05 and fold change (fc) >|1.5|.

Table 2. Summary of selected DEGs in ATG morphant embryos at 72 hpf

Gene name	FC
Interferon, alpha-inducible protein 6	+5.54
Programmed cell death 4 (neoplastic transformation inhibitor)	+3.44
DNA-damage regulated autophagy modulator 1	+3.38
Gamma-glutamyl cyclotransferase	+2.71
Liver-basic fatty acid binding protein mRNA, complete cds.	+2.69
Tripartite motif-containing 35	+2.38
Keratin 20	+2.04
Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	+2.01
Clusterin	+1.97
Interleukin 1, beta	+1.96
Parvalbumin	+1.80
Suppressor of cytokine signaling 3	+1.8
TSC22 domain family, member 3; GRAM domain containing 4	+1.73
Homeobox A11	+1.68
Estrogen receptor 1	+1.68
Very low density lipoprotein receptor	+1.68
ATPase, Na+/K+ transporting, alpha 1 polypeptide	+1.66
Left-right determination factor 2	+1.63
Insulin receptor substrate 2	+1.63
Growth arrest and DNA-damage-inducible, beta	+1.59
Secreted frizzled-related protein 1	+1.58
Insulin-like growth factor binding protein 1	+1.57
BCL2-associated agonist of cell death	+1.56
NudE nuclear distribution gene E homolog (A. nidulans)-like 1	+1.56
Calcium channel, voltage-dependent, gamma subunit 2	+1.56
Myelocytomatosis viral oncogene homolog	+1.55
TNF receptor-associated factor 6	+1.54
Dipeptidyl-peptidase 4	+1.54
Synuclein, beta	+1.52
Tumor necrosis factor receptor superfamily, member 1A	+1.51
Homeobox B5	-1.50
Tyrosyl-tRNA synthetase	-1.51
Mitogen-activated protein kinase 1	-1.52
Chromosome 16 open reading frame 5	-1.54
Homeobox B9	-1.58
Dishevelled, dsh homolog 3	-1.59
Heme oxygenase (decycling) 2	-1.59
Homeobox B3	-1.60

CREB binding protein	-1.60
Apoptosis-inducing factor, mitochondrion-associated, 1	-1.63
Coagulation factor II (thrombin)	-1.64
ADP-ribosylation factor-like 6	-1.64
Pancreatic and duodenal homeobox 1	-1.64
Tumor protein p63	-1.66
Phosphorylase, glycogen, muscle	-1.66
SAFB-like, transcription modulator	-1.69
Toll-like receptor adaptor molecule 1	-1.73
Transferrin	-1.74
Caspase 4, apoptosis-related cysteine peptidase	-1.75
Kalirin, RhoGEF kinase	-1.75
Homeobox B6	-1.76
Homeobox A9	-1.77
Complement component 6	-1.78
Gelsolin (amyloidosis, Finnish type)	-1.8
Growth arrest and DNA-damage-inducible, gamma	-1.83
Jumonji domain containing 6	-2.10
Bardet-Biedl syndrome 7	-2.19
E2F transcription factor 2	-2.28
Solute carrier family 2 (facilitated glucose transporter), member 2	-2.51
Transforming growth factor, beta 1	-2.75
Metallothionein 2	-3.23
Nitric oxide synthase 1 (neuronal)	-3.77

Significantly DEGs are shown at fold change (FC) of ≥1.5 and ≤-1.5 and p-value<0.05.

4. Discussion

In mammals, GLUT2 is a low-affinity, high-capacity sugar transporter that plays an essential role allowing large hexose fluxes in and out of the cells and is an important contributor to the regulation of circulating glucose levels (Uldry and Thorens, 2004). Despite the fact that this carrier has been extensively characterized in mammals, the information available in lower vertebrates still is limited. Thus, in our effort to contribute to our understanding of its physiological role, in this work we describe a vertebrate model of GLUT2 deficiency. Using reverse genetics approaches, we have blocked GLUT2 translation in zebrafish embryos causing a general developmental delay, severe mispatterning of axonal scaffolds and alterations in the development of the neural

progenitor cells. Furthermore, GLUT2 abrogation also caused alterations in the left-right distribution of visceral organs. In addition, we have related these functional consequences of GLUT2 depletion to a reduction in glucose uptake and in an increased in programmed cell death.

Expression analysis by ISH showed that this transporter is expressed in the liver, pronephric tubules, anterior intestine, endocrine pancreas and neurons surrounding the hindbrain region. These data complements our previous data in zebrafish (Castillo et al., 2009) and is consistent with previous data on the localization of the expression of GLUT2 in other teleosts (Panserat et al., 2001; Hall et al., 2006; Polakof et al., 2007; Terova et al., 2009) and in mammals (Thorens et al., 1988; Fukumoto et al., 1989; Bell et al., 1990; Thorens et al., 1990). Between 24 hpf and 48 hpf, GLUT2 mRNA is detected in the brain and pronephric ducts. Interestingly, the pronephric ducts are complete by 24 hours post-fertilization (hpf) (Drummond et al., 1998). Furthermore, the circulation-dependent step of ventricle expansion takes place between 24 hpf, when the onset of the heart beat takes place, and 48 hpf (Lowery and Sive, 2005). Later, at 72 and 120 hpf, an overall increase in GLUT2 mRNA expression is observed, in agreement with our previous study (Castillo et al., 2009). At this time in zebrafish development, GLUT2 expression is observed in intestine, liver, pancreas, pronephric tubules and hindbrain region. Interestingly, the functional maturation of most of the organs conforming the gut is underway by 72 hpf. At this developmental stage, the gut tube is completed and the anterior part of the digestive tract is finished by 74 hpf, when the lumen of the pharynx is visible and the mouth is open (Wallace and Pack, 2003). At this same stage, the budding of the liver is also completed and endothelial cells encapsulate and invade the liver leading to its vascularization (Field et al., 2003). Moreover, by 72 hpf the zebrafish pancreas is already positioned on the right side of the embryo (Biemar et al., 2001), coinciding with the appearance of the pancreatic duct and the secondary pancreatic cells (Tiso et al., 2009). Despite the fact that the production of insulin, glucagon and somatostatin, the major pancreatic hormones, is initiated earlier in development (Biemar et al., 2001; Ober et al., 2003), trypsin expression emerges at 48

hpf, achieving maximum expression levels at 72 hpf (Biemar et al., 2001; Tiso et al., 2009). Trypsin is a marker for mature exocrine cells and its late expression correlates with previous observations in the mouse (Gittes and Rutter, 1992) and with the fact that the requirement for digestive enzymes in zebrafish starts at 96 hpf (Biemar et al., 2001). These evidences could support the hypothesis that GLUT2 appears expressed in the pancreas at this stage as a preparatory event prior to its requirement for glucose-dependent insulin secretion, presumptively needed with the onset of exogenous feeding between 4-5 dpf (Wallace et al., 2005).

From 72 to 120 hpf, we detected the presence of GLUT2 transcripts in the central nervous system (CNS), appearing strongly expressed in the hindbrain, particularly in both corpus cerebelli and medulla oblongata. In mammals, GLUT2 expression has been reported in the cerebellum, brain nuclei, hypothalamic nuclei, neurons glial cells and astrocytes (Leloup et al., 1994; Nualart et al., 1999; Arluison et al., 2004; Arluison et al., 2004; Kang et al., 2004; Roncero et al., 2004; Marty et al., 2005) where it has been suggested to be expressed in glucose-sensing neurons that regulate feeding behavior (Marty et al., 2007). In fish, GLUT2 expression in the brain has been reported in rainbow trout (Polakof et al., 2007), sea bass (Terova et al., 2009) and adult zebrafish (Castillo et al., 2009). Hereby, our results are consistent with our previous data on the localization of GLUT2 expression in adult zebrafish and support the hypothesis of the existence of a glucose-sensing region in the zebrafish brain. Putting these data together, the observed expression of GLUT2 suggests an important role of this transporter in the final steps of gut development and reinforces the idea of GLUT2 as an essential participant in insulin metabolism as well as in glucose sensing in the CNS.

Next, we found that abrogation of GLUT2 expression during early developmental stages resulted in a delay of whole body development with critical consequences in the formation of the CNS. GLUT2 morphant embryos showed less developed eyes and severe alterations in the hindbrain ventricle, affecting the midbrain-hindbrain structures. In view of the coinciding neurodegenerative morphant phenotype and the localization of

GLUT2 expression in the hindbrain at early developmental stages, we examined the expression pattern of a set of known proneural genes. In zebrafish, as in mammals, neurons are classified into two major groups: glutamatergic and GABAergic, depending on their main neurotransmitter (Bae et al., 2009). In the mouse, glutamatergic neurons derived from progenitor cells placed in the upper rhombic lip (URL) express the proneural gene ATOH1 (Alder et al., 1996), while the glutamategic immature and mature granule cells express the proneural gene NEUROD, which is required for their generation and differentiation (Miyata et al., 1999). On the other hand, murine GABAergic neurons are derived from progenitor cells expressing the proneural gene PTF1A (Hoshino et al., 2005; Hoshino, 2006). Consistent with this, it has been recently reported that neurogenic processes of both glutamatergic and GABAergic neurons are conserved between mammals and zebrafish, in which the above-mentioned proneural genes are also specifically expressed in progenitor cells of the hindbrain region (Kani et al., 2010). Thus, we have studied the expression pattern of these proneural marker genes throughout early development in order to find out the effects of the lack of GLUT2 in the hindbrain region. In the case of the marker genes for glutamatergic neurons, NEUROD expression did not appear to be significantly affected in morphant embryos from 24 to 72 hpf. On the other hand, morphant embryos between 24 and 48 hpf evidenced a reduction in ATOH1b expression as well as an alteration in the URL region, but not at 72 hpf. Moreover, ATOH1c did not appear to be affected at 72 hpf.

Strikingly, GLUT2 morphant embryos showed no expression of PTF1a at 24 hpf and at 48 hpf they showed an expression pattern similar to that of 24 hpf control embryos. This suggests that abrogation of GLUT2 may have been affecting the generation of PTF1a-expressing cells from the ventricular zone (VZ) that have been reported in mouse to be the source of all GABAergic neurons in the cerebellum (Hoshino et al., 2005; Hoshino, 2006). In addition, supporting our results, there is recent evidence suggesting that GLUT2 is expressed in mouse GABAergic interneurons and that GLUT2 is regulated by low glucose concentrations in the region of the hindbrain (Sanno et al., 2011). These data are in agreement with the notion that GABA release in

these neurons is regulated by glucose (During et al., 1995; Levin, 2000) and that, when glucose levels decrease under hypoglycemic conditions, it inactivates GABAergic neurons in the ventromedial hypothalamus, enhancing the counterregulatory response to hypoglycemia (Zhu et al., 2010). Hereby, is tempting to hypothesize that in zebrafish, GLUT2 could be expressed in GABAergic neurons as part of the glucosensing capability of the CNS, and that the observed effect on the delayed appearance of progenitor cells expressing ATOH1b in GLUT2 morphant embryos may be a consequence of altered interactions between the two neuronal lineages during their proliferation and differentiation. Furthermore, we confirmed the early alteration of the hindbrain structure by immunostaining of acetylated tubulin, reveling a severe alteration on the neural scaffold at 24 hpf, which is reverted by 48 hpf.

In our studies on the study of the functional consequences of GLUT2 deficiency we were able to demonstrate a significant reduction in the overall glucose uptake in GLUT2 morphant zebrafish embryos and particularly in the cephalic region. Moreover, we showed a decrease in cell viability due to apoptotic cell death, appearing mainly localized in the brain region. These evidences led us to think that the deficiency in glucose uptake caused by abrogation of GLUT2 in zebrafish embryos may result in increased cell death because of the lack of glucose as metabolic fuel. Interestingly, we also observed that morphant embryos presented alterations in the distribution of endoderm-derived tissues. The liver and the exocrine and endocrine pancreas appeared in *situs inversus* position in 55% of the GLUT2 morphants, as assessed using marker genes for each of these organs. GLUT2 expression was observed in these organs, suggesting also that the lack of nutrient availability may be related to alterations in the distribution of GLUT2 expressing tissues. Further analysis with marker genes for intestine and pronephros such as intestine fatty acid binding protein (IFABP) and nephrocystin-4 (NPHP4) will be helpful to address if this effect is extended to all the tissues where GLUT2 is expressed.

Additionally, it is worth mentioning that in order to confirm that all the previously described effects on the zebrafish embryos were specific due to the lack of GLUT2 expression, we performed rescue experiments. Indeed, we rescued the wild-type phenotype in terms of brain structure, glucose uptake and cell viability in morphants with zebrafish and rat GLUT2 RNAs.

Next, to better characterize the changes triggered by the lack of GLUT2 at the transcriptional level, we performed microarray analysis. Interestingly, the results obtained confirmed the knockdown of GLUT2 since this gene appeared highly down-regulated in morphant embryos. Overall, our gene expression data strongly support the described alterations in hindbrain development, glucose metabolism, cell viability and organ patterning. In relation to the observed phenotype in the brain in GLUT2 morphant embryos, the expression of parvalvumin expression was significantly increased. This result supports the idea that GLUT2 abrogation may have affected GABAergic neurons since parvalvumin is a marker gene for these neurons and plays an important role in normal brain functioning (Hashimoto et al., 2003). Furthermore, liver-basic fatty acid binding protein (L-FABP) was up-regulated while pancreatic and duodenal homeobox 1 (PDX1) and transferrin appeared down-regulated. Altered expression of L-FABP and transferrin, two marker genes for liver (Denovan-Wright et al., 2000; Mudumana et al., 2004), confirmed that this organ is affected in morphant embryos. In addition, PDX1 is a marker gene for pancreatic β-cell that is necessary for the proper regulation of the glucose-dependent insulin secretion by β-cells (MacFarlane et al., 1994; Marshak et al., 1996) and has been shown to be necessary for pancreas development in zebrafish since PDX1-null zebrafish lack of this organ (Yee et al., 2001). Hence, the down-regulation of PDX1 expression strongly suggests that the glucose-responsive regulation of insulin synthesis in endocrine pancreas could be affected as a consequence of the abrogation of GLUT2. Interestingly, consistent with this, various genes involved in insulin signaling and glucose metabolism, including insulin receptor substrate 2 and insulin-like growth factor binding protein 1 (IGFBP-1) were up-regulated in GLUT2 morphants. It has been reported that overexpression of IGFBP-1 reduces the growth and

developmental rate in zebrafish embryos (Kajimura et al., 2005), coinciding with our morphant phenotype. Also, ATPase, Na+/K+ transporting, alpha 1 polypeptide and calcium channel, voltage-dependent, gamma subunit 2 were up-regulated. These two genes are directly involved in the depolarization of the plasma membrane in β-cells that leads to the exocytosis of insulin granules triggered by glucose uptake mediated by GLUT2 (Henquin et al., 2003). These data suggests that glucose-dependent insulin secretion may be affected as a consequence of the reduction of glucose uptake in GLUT2-deficient embryos. The reduction in cell viability and the increase in apoptotic cell death assessed *in vivo* by specific staining and immunolocalization, was also confirmed by microarray analysis. The expression of a number of genes involved in apoptotic processes was affected in GLUT2 morphant embryos. Specifically, BCL2-associated agonist of cell death (BAD) appeared up-regulated in GLUT2 morphant embryos. BAD is a member of the largely studied BCL2 family, which is critical for the control of programmed cell death (Scorrano and Korsmeyer, 2003). Furthermore, a link between glucose homeostasis and programmed cell death has previously been established in GLUT1-deficient zebrafish embryos, in which abrogation of BAD rescued the neurodegenerative phenotype (Jensen et al., 2006). Thus, these results suggest that glucose levels may be playing a role in the regulation of cell survival and, therefore, the reduction of glucose uptake due to deficient GLUT2 expression may likely be the cause of the increase in apoptotic cell death.

Our data also showed that the left-right determination factor 2 (LEFTY2) and transforming growth factor beta 1 (TGFβ1) appeared differentially expressed in GLUT2 morphants. Both genes are involved in the regulation of the expression of the Nodal pathway components, required for the normal asymmetric organization of the dorsal diencephalon and endoderm-derived tissues (Bisgrove et al., 1999; Rodaway et al., 1999; Bisgrove et al., 2000). Analysis of mutant zebrafish deficient for two Nodal-type TGFβ ligands, Cyclops and Squint, lack the endoderm (Feldman et al., 1998) and overexpression of Antivin, an inhibitor of the Activin/Nodal signaling pathway related to LEFTY, results in a complete loss of the endoderm (Alexander and Stainier, 1999;

Thisse and Thisse, 1999). Hereby, the observed alteration in LEFTY and TGFβ1 expression could be affecting the formation of endoderm-derived tissues via the Nodal pathway leading to alterations in the left-right asymmetry axis.

To summarize, in the present study we provide evidence for the physiological role of GLUT2 in glucose homeostasis. Importantly, we demonstrate here that GLUT2 is essential for the development of neuronal progenitor cells, especially for the development of the GABAergic neurons, suggesting the existence of a glucose-sensing region in the zebrafish brain in which these neurons may be playing an important role in the regulation of hypoglycemia. Furthermore, our data shows that the defective coordination between glucose uptake and the demand for carbohydrates during early developmental stages in GLUT2 morphant embryos, taken as an indication of deficient nutrient availability, results in increased apoptotic cell death as the underlying cause for the observed alterations in brain development. In view of these results, we propose zebrafish as a useful model for the study of GLUT2-deficient states.

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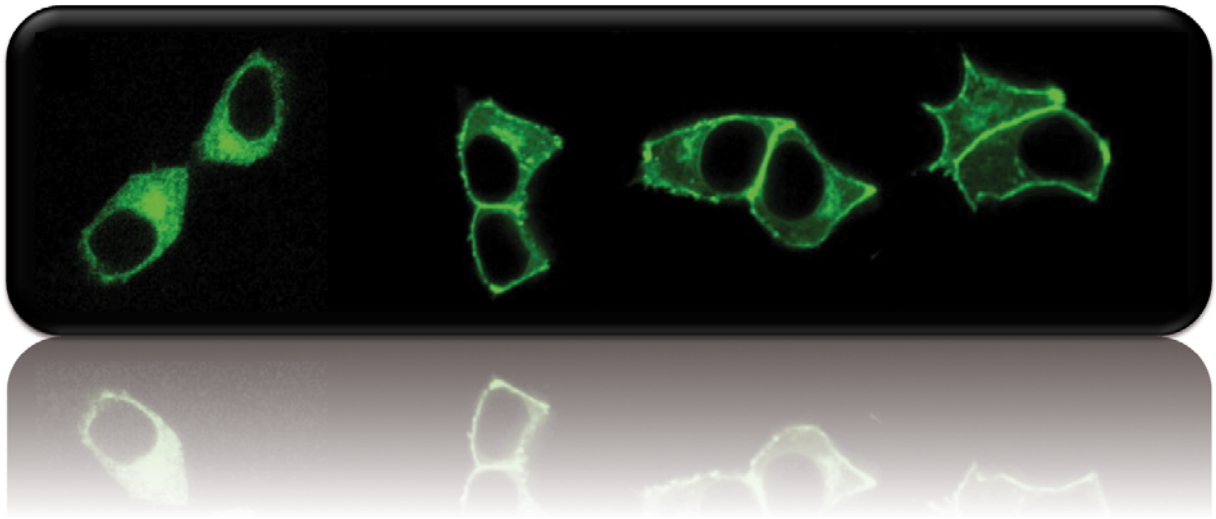
Chapter III

GLUT2:
Establishment of a model to study fish GLUT2 function and regulation in mammalian pancreatic cells



"Science is a way of trying not to fool yourself. The first principle is that you must not fool yourself, and you are the easiest person to fool"

Richard Feynman

1. Introduction

Insulin plays a vital role regulating glucose homeostasis in higher vertebrates and glucose uptake represents the initial step in glucose stimulated insulin secretion by pancreatic β-cells (Kahn, 1996; Guillam et al., 2000). In mammals, GLUT2 has been shown to be the most important glucose transporter expressed in pancreatic β-cells, being the only GLUT found to the date in murine β-cells (Guillam et al., 2000).

GLUT2 is a low-affinity, high-capacity glucose transporter with the highest *Km* for glucose (17 mM) among the known members of GLUT family (Johnson et al., 1990). It is expressed at a very high level in pancreatic β-cells, specially in the microvilli present in the lateral membrane (Orci et al., 1989). GLUT2 cell surface expression in β-cells is responsible for glucose uptake, which will trigger glucose-induced insulin secretion (Ohtsubo et al., 2005). The entry of glucose through GLUT2 leads to plasma membrane depolarization through the closure of ATP-dependent K^+ channels and the influx of Ca^{2+} to exocytose insulin granules (Henquin et al., 2003). In β-cells, GLUT2 equilibrates extra- and intracellular glucose concentrations providing an unrestricted supply of glucose, being glucokinase the rate-limiting step in glucose entry (Matschinsky, 2002). Furthermore, it has been reported that in β-cells engineered with different GLUT isoforms, only GLUT2 allows for normal insulin production in response to glucose stimulation (Hughes et al., 1993). In addition, decreased expression of GLUT2 has been shown to be linked with the loss of glucose-stimulated insulin secretion in a number of animal models of type 2 diabetes (Unger, 1991; Valera et al., 1994; Guillam et al., 1997).

In teleost fish, GLUT2 has been characterized in several species (Wang et al., 1994; Krasnov et al., 2001; Hall et al., 2006; Castillo et al., 2009; Terova et al., 2009). However, to the date, expression in the fish pancreas has been described only in rainbow trout (Polakof et al., 2007) and in zebrafish (Chapter 2). Functionally, zebrafish GLUT2 has an affinity for 2-DG of 11 mM, in a similar range to the mammalian constant (Castillo et al., 2009). Moreover, zebrafish GLUT2 also allows the transport of mannose,

fructose and galactose, as well as L-glucose but with low affinity (Castillo et al., 2009), as observed in mammals (Cheeseman, 2002). These properties indicate a high degree of functional conservation between fish GLUT2 and its mammalian homolog, extending the notion that GLUT2 is a low-affinity, high-capacity glucose transporter (Uldry and Thorens, 2004) throughout vertebrates, from fish to mammals.

Regarding the physiological role of GLUT2, little is known about its involvement in glucose homeostasis and insulin action or secretion in fish. Interestingly, previous studies investigating the modulation of GLUT2 expression demonstrated that mRNA expression levels in the liver are not affected in fasting/re-feeding conditions (Panserat et al., 2001; Hall et al., 2006). However, our group has recently shown that GLUT2 expression in zebrafish is under nutritional regulation in the intestine (Castillo et al., 2009). Furthermore, GLUT2 expression in the rainbow trout pancreas decreased under hypoglycemic conditions and increased under hyperglycemic conditions (Polakof et al., 2007), similar to that reported in mammalian pancreatic β-cells (Mueckler, 1994).

To better understand the physiological role of GLUT2 in zebrafish, in this study we aimed to establish an *in vitro* system using the pancreatic β-cell line MIN6 (Miyazaki et al., 1990). MIN6 is an insulinoma cell line derived from a transgenic mouse, that presents glucose-dependent insulin secretion resembling normal pancreatic β-cells and constitutes a useful tool for studying the mechanism of glucose-stimulated insulin secretion (Miyazaki et al., 1990; Ishihara et al., 1993). In this study, we set out to test the hypothesis that zebrafish GLUT2, when expressed in MIN6 cells in which endogenous GLUT2 has been abrogated, could reconstitute glucose-induced insulin secretion and, therefore, demonstrat the functionality of zebrafish GLUT2. First, we attempted to block the expression of the endogenous GLUT2 by transfecting MIN6 cells with short interfering RNAs (siRNAs) specifically designed against mouse GLUT2. Next, we analyzed the functional consequences of abrogating the expression of endogenous GLUT2 in MIN6 cells by measuring the secretion of insulin in response to glucose stimulation. Furthermore, we performed overexpression assays by transfecting expression constructs containing rat or zebrafish GLUT2 and their functionality was

assessed examining glucose uptake in cells expressing these constructs. The results from our study indicate that we have found an effective dose to knock down the endogenous GLUT2 in MIN6 cells leading to a loss of glucose-dependent insulin secretion. Moreover, we show that overexpression of zebrafish GLUT2 in wild type MIN6 results in a significant increase in glucose uptake. Therefore, in this study we have provided proof of concept that MIN6 cells are suitable for studying the functional role of zebrafish GLUT2 in pancreatic β-cells. However, experimental conditions will require to be optimized in future studies in order to provide solid evidence for a functional role of zebrafish GLUT2 in glucose-stimulated insulin secretion.

2. Materials and methods

Materials

MIN6 were kindly provided by Dr. Albert Barberà (IDIBAPS, Barcelona, Spain). Dulbecco's modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and other tissue culture reagents were purchased from Sigma Aldrich (Madrid, Spain). The radiolabeled glucose analog 2-deoxy-D-[^{3}H] glucose (2-[^{3}H] DG) was purchased from Perkin Elmer (Madrid, Spain). Polyclonal anti-GLUT2 antibody was obtained from Millipore (Madrid, Spain). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and anti-mouse IgGs were from Cell Signaling (Barcelona, Spain). Monoclonal anti-β-actin antibody was purchased from Sigma Aldrich (Madrid, Spain).

Generation of expression constructs

The rat Myc-GLUT2- GFP vector was kindly donated by Dr. Jeffrey Pessin (Albert Einstein School of Medicine, NY, USA) (Hou et al., 2009). The zebrafish GLUT2 cDNA was obtained from a whole-body zebrafish cDNA library as previously described (Castillo et al., 2009) and its full-length sequence was amplified by PCR and subcloned in pcDNA3 vector containing EGFP. All constructs were verified by DNA sequencing.

Maintenance of MIN6 cells

MIN6 cells were maintained at 37ºC (95% O_2/5% CO_2) in DMEM supplemented with 15% heat inactivated FBS and 100 U/ml penicillin/0.1 mg/ml streptomycin. Medium was changed every 2–3 days. MIN6 cells used in the present study were harvested at passages 31-38.

Transient transfection and siRNA treatment of MIN6 cells

Approximately 1 x 10^5 cells/well were plated in 12 well plates and transfected 24 h later at a confluence of 70-80% with Lipofectamine 2000 (Invitrogen, Prat del Llobregat, Spain) following the manufacturer's indications. For the knockdown assays, MIN6 cells were transfected with a set of 4 different ON-TARGETplus mouse GLUT2 siRNAs from Dharmacon (Lafayette, CO, USA), following the manufacturer's indications. siRNAs were titrated and transfected at 75, 125, 200 and 300 nM of final concentration. Cells were stimulated and/or lysed at 48 h post-transfection. Overexpression experiments were performed by transfecting 2 μg/well of rat-myc-GLUT2-GFP or zfGLUT2-GFP constructs. Cells were stimulated and/or lysed 24 h post-transfection. MOC controls were transfected only with lipofectamine and cultured in the same conditions as other transfected cells.

Insulin secretion and radioimmunoassay

MIN6 cells were first incubated for 2 h in HEPES-buffered Krebs Ringer (KRBH) solution (125 mM NaCl, 4.7 mM KCl, 1 mM CaCl2, 5 mM $NaHCO_3$, 1.2 mM MgSO4, 1.2 mM KH_2PO_4, 25 mM HEPES) containing 0.5% bovine serum albumin (BSA). Cells were then incubated with KRBH-BSA containing 33.4 mmol/l glucose for 2 h. Supernatants were then collected, stored at -80ºC for insulin determination and cells were lysed in RIPA buffer supplemented with a protease inhibitor cocktail purchased from Sigma Aldrich (Madrid, Spain) for immunoblotting. Radioimmunoassays were performed with a commercial kit from MP Biomedical (Solon, OH, USA) using rat insulin as the standard.

Glucose Uptake Assays

MIN6 cells were cultured as described above. Cells were washed twice with HEPES-buffered saline (HBS) and incubated with HBS containing 50 μM 2-deoxyglucose [0.5 μCi/ml 2-[^{3}H]-deoxyglucose (2-[^{3}H]-DG)] for 30 min at room temperature. After this period, the transport solution was removed and cells were rinsed three times with ice-cold PBS containing 50 mM D-Glucose. Finally, cells were lysed with 0.05 N NaOH, and the radioactivity was determined by scintillation counting using a β-counter (Packard Bioscience, Meriden, CT). Protein concentration was measured by the Bradford method (Bradford, 1976). Nonspecific uptake was carried out in the presence of 50 μM cytochalasin B in the transport solution, and these values were subtracted from all other values. Glucose uptake measured in triplicate and normalized to total protein was expressed as fold induction with respect to non stimulated cells.

Immunoblotting

Western blot analyses were conducted using lysates from MIN6 cells (as described above). Lysates (10 μg protein) were heated for 5 min at 60°C and samples and protein standards Precision Plus from BioRad (Barcelona, Spain) were loaded and separated on 10 or 12% SDS-PAGE gels using a Mini-Protean system (BioRad, Madrid, Spain) for 1–2 h at 100 V and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Madrid, Spain). The membrane was blocked overnight with TBST buffer (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.6) containing 5% (w/v) non-fat dry milk. Next, the membrane was washed several times in TBST and incubated overnight at 4°C with anti-GLUT2 diluted to 1:5000 in TBST under continuous shaking. After three washes, the membrane was incubated with a secondary goat anti-rabbit-HRP diluted to 1:15000 in TBST. Immune complexes were detected by chemiluminescence using Supersignal West Pico detection kit from ThermoScientific (Madrid, Spain), according to the manufacturer's instructions and visualized with a luminescent image analyzer (FujiFilm LAS-3000). For normalization, we used a anti-β-

actin antibody. Bands were quantified using ImageJ 1.45 software (National Institutes of Health, NIH).

Statistical analysis

Results are expressed as mean ± SE. Statistical differences were analyzed by Kruskal-Wallis and Mann-Whitney non-parametric tests and considered to be significant at $p \leq 0.05$.

3. Results

Insulin secretory properties of MIN6 cells

In order to confirm the functional properties of the MIN6 cells, we measured their ability to secrete insulin in response to glucose stimulation. Our data showed that the stimulation of the cells with 33.4 mM D-glucose for 2 h led to a 2.5 -fold increase in insulin secretion (Fig.1). This result evidenced that the cells presented glucose-dependent insulin secretion, being consistent with previous data (Ishihara et al., 1993).

Table 1. Sequences of the siRNA duplexes tested in the knockdown experiments.

siRNA	Sequence location	Target sequence (5´→3´)
siRNA 1	3' UTR	UGGAUUAGACUUCGGGUUU
siRNA 2	3' UTR	CCGAUUAGAAAUAUCAACA
siRNA 3	3' UTR	UAAUAAACUGGGUCAAUCA
siRNA 4	ORF	GCUUUGCAGUGGACGGAAU

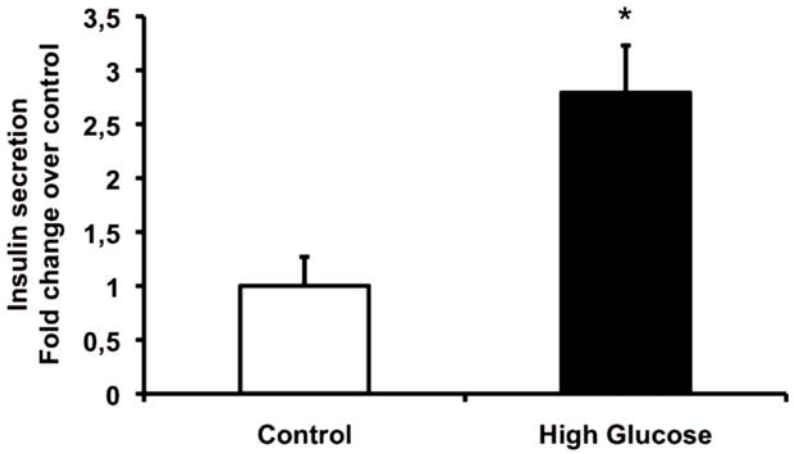


Figure 1. Insulin secretion by MIN6 cells. MIN6 cells were first incubated in KRBH-BSA for 2 h and then incubated in KRBH-BSA solution in the absence (Control) or presence of 33.4 mM D-glucose for 2 h (High Glucose). Data are shown as fold change in insulin secretion compared to the non-stimulated cells. Each column represents the mean ± S.E. of six measurements derived from 3 independent experiments. * indicates statistical significant differences with respect to the non-stimulated cells ($p<0.05$).

Knockdown of endogenous GLUT2 by siRNA

To knockdown GLUT2 in MIN6 cells we tested 4 different commercial candidate siRNAs (Table 1). MIN6 cells were transfected with individual ON-TARGETplus duplexes specifically designed for mouse GLUT2. Candidate siRNAs were transfected at different concentrations (from 75 to 300 nM), observing consistent and significant reduction of 13% of GLUT2 protein levels only when using the siRNA 4 at a final concentration of 200 nM (Fig. 2). To assess whether the knockdown of GLUT2 was exerting an effect on glucose-dependent insulin secretion, we treated MIN6 cells transfected with siRNA 4 with D-glucose at a concentration of 33.4 mM. MOC control cells significantly increased insulin secretion in response to glucose; however, siRNA 4-transfected cells did not secrete insulin in response to glucose (Fig. 3). Therefore, our data confirmed that the knockdown of GLUT2 was affecting insulin secretion in transfected MIN6 cells

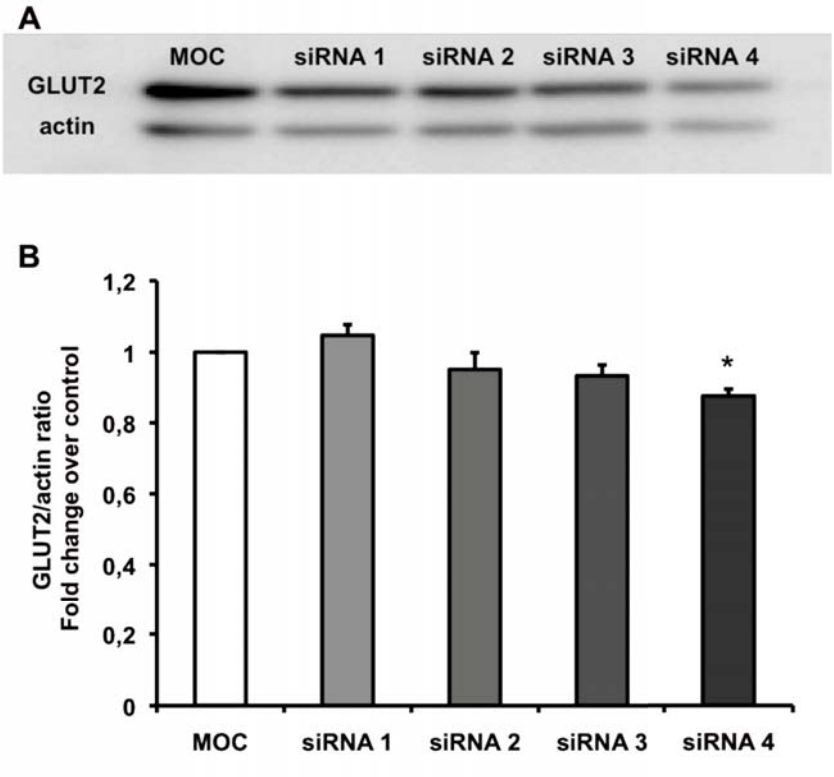


Figure 2. Effects of different candidate GLUT2 siRNAs on GLUT2 protein levels in MIN6 cells. siRNAs were used at a final concentration of 200 nM and cells were lysed and collected at 48 h post-transfection. (A) Representative immunoblots of GLUT2 and actin proteins in lysates of MIN6 cells transfected with the candidate siRNA duplexes. 10 µg of protein were loaded in each lane. (B) Densitometric analysis of GLUT2/actin ratios from MIN6 cells transfected with the candidate siRNAs. Each column represents the mean ± S.E. of six measurements derived from 3 independent experiments in the case of MOC and siRNA 4, and of four measurements derived from two independent experiments in the case of siRNA1, 2 and 3. * indicates statistical significant differences with respect to the MOC cells ($p<0.05$).

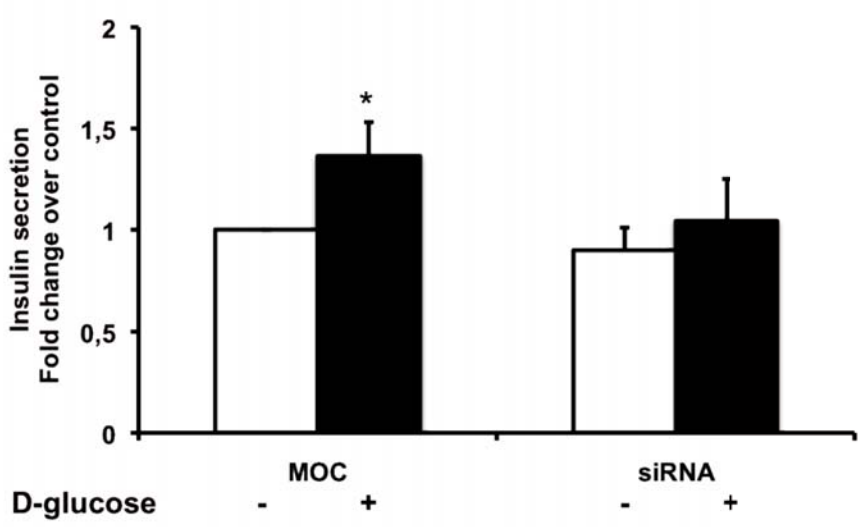


Figure 3. Insulin secretion by MIN6 cells transfected with siRNA 4. MIN6 cells were transfected with the siRNA 4 at a final concentration of 200 nM. For stimulation, cells were first incubated for KRBH-BSA for 2 h and then incubated with KRBH-BSA solution containing 33.4 mM D-glucose for 2 h. Non-stimulated cells were maintained in KRBH-BSA without D-glucose. KRBH-BSA media were collected at 48 h post-transfection. Data are shown as fold change of insulin secretion compared to MOC control cells. Each column represents the mean ± S.E. of six measurements derived from 3 independent experiments. * indicates statistical significant differences with respect to the MOC cells ($p<0.05$).

Overexpression assays with rat and zebrafish GLUT2

To determine the functionality of the rat and zebrafish GLUT2 constructs by overexpressing rat or zebrafish GLUT2, we analyzed the basal secretion of insulin in MIN6 cells transfected with increasing concentrations of these plasmids (from 2 to 6 µg) (Fig. 4). For the two constructs, the results obtained suggested a strong reduction in basal insulin secretion in cells transfected with 4 and 6 µg of the plasmids. In view of this, and based on previous studies using the rodent construct (Hou et al., 2009), we chose 2 µg as the dose for subsequent experiments. Next, we aimed to investigate the glucose-stimulated insulin secretion levels in rat and zebrafish GLUT2-overexpressing cells. The basal secretion of insulin was similar between control and transfected cells and glucose also caused a similar increase (although statistically not significant) in insulin secretion in all three groups (Fig. 5).

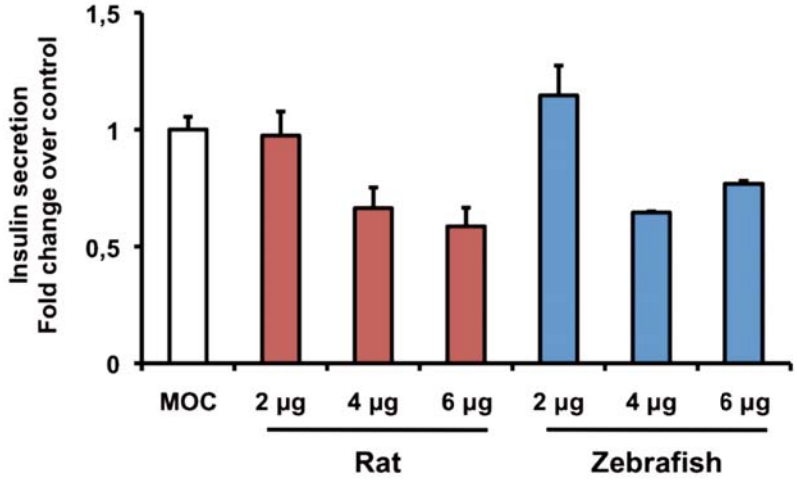


Figure 4. Insulin secretion by MIN6 cells transfected with different plasmid concentrations. MIN6 cells were transfected with 2, 4 and 6 µg of the two constructs used: rat-myc-GLUT2-GFP (Rat) and zebrafish-GLUT2-GFP (Zebrafish). KRBH-BSA media were collected at 24 h post-transfection. Data are shown as fold change of insulin secretion compared to MOC control cells. Each column represents the mean ± S.E. of two measurements derived from 1 experiment.

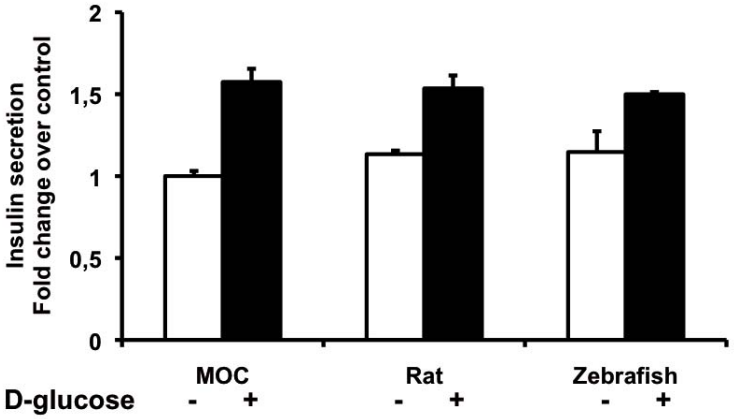


Figure 5. Insulin secretion by MIN6 cells overexpressing rat and zebrafish GLUT2. MIN6 cells were transfected with 2 µg of either construct: rat-myc-GLUT2-GFP (Rat) or zebrafish-GLUT2-GFP (Zebrafish). For stimulation, cells were first incubated in KRBH-BSA for 2 h and then incubated with KRBH-BSA containing 33.4 mM D-glucose for 2 h. Non-stimulated cells were maintained in KRBH-BSA without D-glucose. KRBH-BSA media were collected 24 h post-transfection. Data are shown as fold change of insulin secretion compared to MOC control cells. Each column represents the mean ± S.E. of two measurements derived from 1 experiment.

Subsequently, to further study the functionality of the overexpressed rat and zebrafish GLUT2, we examined glucose uptake under basal conditions in MIN6 cells transfected with the rat and the zebrafish constructs. The results obtained indicated that cell transfected with the zebrafish GLUT2 showed a significant increase in glucose uptake (1.5 fold over control). However, MIN6 cells expressing the rat construct, although showing an upward trend, did not show significant differences in glucose uptake compared to control cells, probably due to the large variability among experiments (Fig. 6).

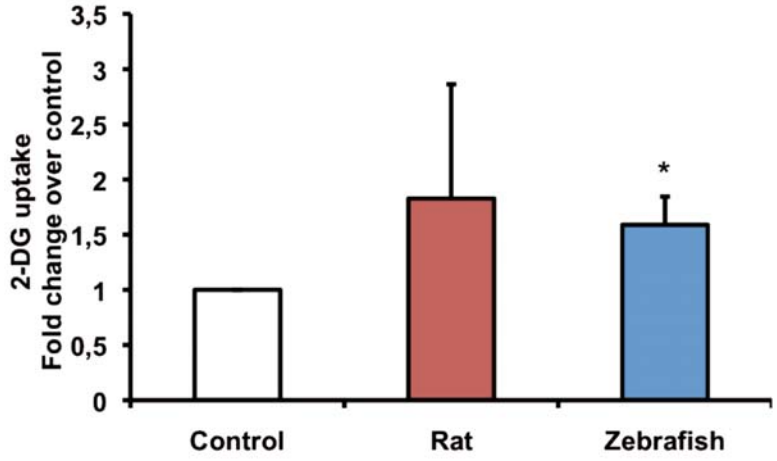


Figure 6. Effects of overexpression of rat and zebrafish GLUT2 on glucose uptake in MIN6 cells. MIN6 cells were transfected with 2 µg of either construct: rat-myc-GLUT2-GFP (Rat) or zebrafish-GLUT2-GFP (Zebrafish), and 2-DG glucose uptake was determined at 24 h post transfection. Data are shown as fold change of glucose uptake compared to control cells. Each column represents the mean ± S.E. of at least six measurements derived from 3 independent experiments. * indicates statistical significant differences with respect to the control cells ($p<0.05$).

4. Discussion

In higher vertebrates, it has been extensively demonstrated that pancreatic β-cells are responsible for the synthesis and release of insulin granules as a consequence

of cellular depolarization through changes in the cellular ATP:ADP ratio (Deeney et al., 2000; Henquin et al., 2003; Thorens, 2011). In the postprandial state, elevation of circulating glucose is sensed in β-cells by the GLUT2 facilitative glucose transporter and the hexokinase isoform glucokinase, leading to the above-mentioned changes in the membrane and triggering the secretion of insulin (Deeney et al., 2000). Furthermore, several states of impaired glucose-stimulated insulin secretion result from a decrease in GLUT2 expression levels. Aging and states of hyperglycemia have been associated with a decline in GLUT2 expression levels, whereas insulin resistance caused by a high fat diet results in impaired plasma membrane targeting of GLUT2 (Laybutt et al., 2002; Reimer and Ahren, 2002; Ohtsubo et al., 2005). The importance of GLUT2 in whole-body glucose homeostasis has been established in GLUT2 null mice by showing that they are hyperglycemic and hypoinsulinemic, having lost the glucose sensing capability necessary for insulin secretion (Guillam et al., 1997; Thorens et al., 2000). Interestingly, rescue of murine GLUT2 null pancreatic β-cells by re-expressing GLUT2 with recombinant lentiviral constructs restored the glucose-dependent insulin secretion (Guillam et al., 2000).

In view of these evidences, in this work we aimed to establish an *in vitro* system for investigating the functional properties of zebrafish GLUT2 using the murine β-cell line MIN6. MIN6 cells show glucose-stimulated insulin secretion similar to that of normal islet cells (Ishihara et al., 1993). Therefore, MIN6 cells constitute a suitable heterologous model system in which to examine the properties of zebrafish GLUT2 regarding its ability to restore the normal physiological characteristics of GLUT2-deficient MIN6 cells. In order to pursue this objective, we set out to establish the experimental conditions to express constructs containing zebrafish GLUT2, using rat GLUT2 as a control, in the murine β-cell line MIN6 lacking the endogenous GLUT2 by siRNA knockdown. Here we show that we have been able to knock down the endogenous GLUT2 in MIN6 cells, leading to a loss of glucose-stimulated insulin secretion. In addition, we evidenced that MIN6 cells expressing the zebrafish GLUT2 construct significantly increased the transport of glucose.

Making use of the MIN6 cell line, we first searched for the proper conditions to abrogate GLUT2 expression in these cells by using different siRNAs. Thus, we showed a reduction of GLUT2 protein levels in MIN6 cells transfected with siRNA 4 at a dose of 200 nM. In agreement with this result, we confirmed the loss of the ability to secrete insulin in response to glucose in knocked down cells. Despite these positive results, it is clear that a higher reduction of GLUT2 protein levels will be desirable in order to establish a tractable system. In this light, future experiments combining the different siRNAs available or designing more effective ones may be required for a more efficient knock down of endogenous GLUT2 in MIN6 cells.

Next, we performed overexpression experiments in order to establish the conditions for the subsequent rescue of GLUT2-deficient MIN6 cells. In our experiments, we observed a dose-dependent inhibitory effect on glucose induced-insulin secretion, where both constructs, at final concentrations above 2 µg/well, caused a reduction in insulin secretion, maybe due to a toxic effect. Previous studies reported that successful transfection of GLUT1, GLUT2 and GLUT4 was performed at the dose of 2 µg/well (Hou et al., 2009). However, no differences in insulin secretion were observed between cells expressing the mammalian and fish constructs and control cells. This could be due to the fact that at 33.4 mM MIN6 cells are almost at the maximum of their responsiveness (Ishihara et al., 1993).

Interestingly, we found that glucose uptake in cells expressing zebrafish GLUT2 was significantly increased, confirming that this transporter is able to contribute to the glucose transport in a mammalian pancreatic cell. Furthermore, these results are consistent with previous data showing that the cloned zebrafish GLUT2 was successfully expressed, functional and characterized in terms of its sugar transport properties in *Xenopus* oocytes (Castillo et al., 2009). On the other hand, cells expressing rat GLUT2 construct did not show a significantly increase in glucose uptake but showed higher average values than controls although with a large variability among experiments. Further work is needed to clearly show that the zebrafish and rat GLUT2 are correctly expressed in MIN6 cells and functional.

To summarize, in this work we present the first steps for the establishment of an *in vitro* model for the study of the function and regulation of zebrafish GLUT2. Future experiments will be focused on the search for a more efficient knockdown of endogenous GLUT2 in MIN6 cells, and the consequent rescue experiments with both rat and zebrafish constructs. In addition, in vivo and in vitro immunolocalization in rescued cells will be performed taking the advantage of the GFP protein fused to GLUT2 in the constructs. Hereby, despite the fact that further optimization of this cell model is needed, this MIN6-based system will allow us to analyze the functionality of the zebrafish GLUT2. This will represent a valuable tool for the study of the physiological role of GLUT2 since there is a high conservation of structure and functional characteristics between fish and mammals.

Acknowledgements

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General Discussion

The aim of this thesis was to study the function and regulation of two of the major players in the carbohydrate metabolism regulated by insulin, the facilitative glucose transporters GLUT2 and GLUT4, in teleost fish. In non-mammalian vertebrates, GLUT2 has been poorly characterized to date. It has been shown that in various teleost species GLUT2 is expressed in the main insulin sensitive tissues (Krasnov et al., 2001; Hall et al., 2006; Polakof et al., 2007; Castillo et al., 2009; Terova et al., 2009), similar to what is described in mammals (Joost and Thorens, 2001; Uldry and Thorens, 2004). However, the functional properties and physiological role of GLUT2 have been poorly characterized in fish. In view of this, we have characterized GLUT2 in zebrafish as it is one of the most recognized models for the study of physiology, development and metabolism (Briggs, 2002; Gerhard, 2003; Schlegel and Stainier, 2007). Regarding GLUT4, our group has pioneered the research of this transporter in teleost species since Planas and co-workers characterized the first GLUT4 homolog in a lower vertebrate (Planas et al., 2000). Despite the fact that more attention has been placed in the study of this glucose transporter than in GLUT2, information regarding the factors involved in the regulation of the transcription of the GLUT4 gene have been poorly characterized in mammals, while no data is available in lower vertebrates. For this purpose, in this study we have analyzed the regulation of a teleost GLUT4 promoter under stimulus known to modulate GLUT4 transcription and expression in mammals, such as insulin (Cooke and Lane, 1998; Cooke and Lane, 1999; Giron et al., 2008), fiber contraction (Slentz et al., 1992; MacLean et al., 2002; Silva et al., 2005; Lima et al., 2009) and PPARs (Armoni et al., 2003). Thus, the major findings obtained from these studies according to the proposed objectives are listed bellow:

To study the transcriptional regulation of the GLUT4 gene in teleost fish by identifying and characterizing a functional promoter region of the Fugu (Takifugu rubripes) GLUT4 gene.

In mammals, GLUT4 plays a pivotal rate-limiting role in the insulin-stimulated glucose uptake in skeletal and cardiac muscle types and in white and brown adipose tissues (Huang and Czech, 2007). Insulin has been largely studied as the main

modulator of this transporter because of its direct implication in pathologies of impaired glucose uptake and insulin resistance such as type 2 diabetes and obesity (Kusari et al., 1991; Shepherd and Kahn, 1999; Friedel et al., 2002; Karnieli and Armoni, 2008). However, GLUT4 is also under the regulation of other factors, namely muscle contraction, when extra fuel in the form of glucose is required by the cells (Lauritzen and Schertzer, 2010).

GLUT4 in fish is also regulated by the action of factors related to glucose metabolism and insulin-stimulated glucose uptake. A number of studies describe how fish GLUT4 is regulated in muscle and adipose tissues at the levels of RNA expression, protein synthesis and translocation to the plasma membrane (Capilla et al., 2002; Capilla et al., 2004; Hall et al., 2006; Diaz et al., 2007; Diaz et al., 2007; Diaz et al., 2009; Capilla et al., 2010; Polakof et al., 2010; Vraskou et al., 2011). However, there is no information regarding the mechanisms that govern the regulation of the transcription of the GLUT4 gene in teleosts. In order to investigate the role of factors exerting a control on the transcription of the GLUT4 gene, we successfully cloned the GLUT4 promoter in Fugu. The 5´-flanking region of the Fugu GLUT4 gene showed similar features to that in mammals: i.e. multiple TSSs, no TATA-box elements, GC-rich regions close to the core promoter and several binding sites for nuclear transcription factors including SP1 and C/EBP (Kaestner et al., 1990; Buse et al., 1992; Liu et al., 1992; Richardson and Pessin, 1993). Structurally, comparative analysis between the cloned promoter sequence and that of other fish promoters revealed a high degree of conservation among teleost species and clearly evidenced that the Fugu GLUT4 promoter presents two highly conserved regions, one of which contains the E-box/MEF2/Klf cassette. This cassette is also present in the mammalian GLUT4 promoters (Zorzano et al., 2005), where it plays an important role in the tissue-specific expression of GLUT4 (Liu et al., 1994; Olson and Pessin, 1995; Thai et al., 1998).

Furthermore, we demonstrated the functionality of the cloned fugu GLUT4 promoter, showing a basal activity 2-fold higher than the rat GLUT4 promoter when expressed in the mammalian skeletal muscle cell line L6. Additionally, by generating

several deletion constructs we were able to determine the minimal promoter, with the core promoter being located between nucleotides -132 and +93.

To describe the transcriptional regulation of the Fugu glut4 gene by insulin, a PPARγ agonist and contractile stimuli in skeletal muscle cells.

GLUT4 is regulated at different levels by a vast number of factors and, although GLUT4 has received extensive experimental attention, the mechanisms through which insulin and other mediators exert their action on GLUT4 are not completely understood (Thorens and Mueckler, 2010). It is known that physiological states of altered glucose homeostasis lead to variations in GLUT4 mRNA levels in muscle and adipose tissue. Specifically, the expression of this transporter in skeletal muscle has been reported to increase with muscle contraction resulting from physical exercise and to decrease during states of insulin deficiency, including diabetes type II and obesity (Ren et al., 1994; Kawanaka et al., 1997; Host et al., 1998; Thorens and Mueckler, 2010). Therefore, the major focus of this work was to improve our understanding of the molecular processes regulating glucose homeostasis by unraveling the mechanisms involved in the regulation the transcription of the GLUT4 gene.

As mentioned above, insulin stimulates GLUT4 expression, protein synthesis and translocation to the cell surface in mammals (Bryant et al., 2002; Ishiki and Klip, 2005; Larance et al., 2008) as well as in fish (Capilla et al., 2004; Diaz et al., 2007). However, the effect exerted by this hormone on GLUT4 at the transcriptional level has been poorly characterized to date. In this study, we have observed that the activity of the Fugu GLUT4 promoter is inhibited by insulin in a dose- and time-dependent manner, consistent with previous evidences in mammals (Cooke and Lane, 1998; Cooke and Lane, 1999; Giron et al., 2008). Furthermore, all the deletion constructs were repressed by insulin, suggesting the presence of regulatory elements downstream of the TSS. These results lead us to hypothesize that perhaps the cloned GLUT4 Fugu promoter that we studied may have not contained the region(s) necessary for the transcriptional

activation of GLUT4 by insulin. In this regard further studies will be needed to identify those regions.

Next, we investigated the effects of PPARγ activation in L6 murine muscle cells transfected with the cloned Fugu GLUT4 promoter. PPARs are ligand–activated transcription factors from the nuclear receptor family (Fajas et al., 2001). Amongst the three PPAR isoforms (α, β and γ), PPARγ is mainly expressed in the insulin-sensitive tissues, such as muscle and adipose tissue, and plays an important role in the regulation of lipid metabolism and glucose homeostasis (Fajas et al., 1997; Olefsky and Saltiel, 2000). Therefore, we studied the effects of PG-J2, a known endogenous PPARγ agonist (Forman et al., 1995; Kliewer et al., 1995), as a possible modulator of the transcription of the Fugu GLUT4 gene. Our results showed that stimulation with PG-J2 significantly stimulated the activity of the Fugu GLUT4 promoter and that this effect was abolished in the Fugu GLUT4 promoter deletions lacking the PPAR/RXR motifs. This result is in agreement with the fact that PPARγ receptors regulate transcription by forming heterodimes with RXR (Armoni et al., 2003).

Finally, we investigated the in vitro effects of experimentally controlled muscle fiber contraction using C2C12 contractile cells expressing the construct containing the Fugu GLUT4 promoter. Using this system to mimic the effects of exercise *in vitro* in differentiated myotubes we showed an increase in the transcriptional activity of the Fugu GLUT4 promoter. Our findings also suggest the importance of the binding motif HIF-1 for the transcriptional activation of the Fugu GLUT4 promoter in response to muscle contraction since all the HIF-1-containing constructs significantly increased their transcription rate in response to electrical pulse stimulation, consistent with the literature in mammals (Silva et al., 2005; Lima et al., 2009). Taken together, these results clearly indicate that muscle contraction may be regulating the transcription of the GLUT4 gene and suggest the implication of HIF-1 as a modulator of GLUT4 transcription in hypoxic conditions derived from physical exercise (Wagner, 2001).

To study the localization of GLUT2 expression in zebrafish embryos throughout early developmental stages.

GLUT2 is the member of the SLC2 family with the ability to transport the highest amounts of dietary sugars, mostly glucose, and is expressed in the main tissues involved in the handling of carbohydrates (i. e. liver, endocrine pancreas, etc.) (Bell et al., 1990; Thorens and Mueckler, 2010). Due to its low affinity and high capacity, this glucose transporter is able to handle large fluxes of sugars, equilibrating glycemic levels in and out the cells. Moreover, GLUT2 is the main glucose transporter expressed in pancreatic β-cells and mediates the glucose-induced secretion of insulin (Henquin et al., 2003). Previous studies on GLUT2 in fish indicate that the tissue distribution and functional properties of this glucose transporter are similar to that in mammals (Castillo et al., 2009). Nevertheless, relatively little is known regarding the physiological role of GLUT2 in lower vertebrates. In teleost fish, GLUT2 is known to be under the regulation of plasma glucose in the hindbrain and pancreas (Polakof et al., 2007) and under the nutrient availability in the intestine. However, in fasting/re-feeding experiments data are contradictory to those available in mammals, since GLUT2 expression is not altered in the liver in rainbow trout and Atlantic cod (Hall et al., 2006) and is inversely regulated in the zebrafish intestine (Castillo et al., 2009). Apart from these differences in the nutritional regulation of GLUT2 expression that could be attributed to species-specific differences in GLUT2 regulation or function, little is know regarding the actual function of GLUT2 during early development. For this reason, we studied the localization of GLUT2 expression during early development and the effects of abrogation of GLUT2 in the zebrafish embryo.

The expression pattern of GLUT2 during embryonic development in zebrafish was studied by ISH, observing transcripts in the liver, pronephric tubules, anterior intestine, endocrine pancreas and neurons surrounding the hindbrain region. The expression in the pronephric system and in the brain was detected early in development, from 24 hpf and onwards. GLUT2 mRNA was first detected in the liver, anterior intestine and endocrine pancreas at 72 hpf coinciding with the completion of the gut (Wallace and

Pack, 2003). Also at this point, the kidney expression of GLUT2 was mainly localized in the pronephric tubules and the expression in the brain was located mainly in the hindbrain, both in the corpus cerebelli and medulla oblongata. These results support the notion of a glucose-sensing region in the zebrafish brain as hypothesized in rainbow trout (Polakof et al., 2007) and in mammals (Marty et al., 2007).

To summarize, these findings are consistent with the observed distribution in other teleost fish (Panserat et al., 2001; Hall et al., 2006; Polakof et al., 2007; Castillo et al., 2009; Terova et al., 2009) and in mammals (Thorens et al., 1988; Fukumoto et al., 1989; Bell et al., 1990; Thorens et al., 1990) and highlight the importance of GLUT2 for the development of endoderm-derived tissues and reinforce the idea of the existence of a glucose-sensing unit in the zebrafish brain.

To evaluate the effects of abrogating zebrafish GLUT2 with antisense morpholinos on embryonic development and to describe the functional alterations caused by the GLUT2 knockdown.

A number of studies have resorted to the use of GLUT2-deficient organisms to analyze the function of this transporter. GLUT2-null mice show early deficient insulin secretion, presenting a diabetic phenotype, and only survive if are fed with low-sugar pellets (Guillam et al., 1997). Furthermore, GLUT2 knockout mice present hyperglycemia and high levels of free fatty acids in plasma, glucosuria and abnormal feeding behavior (Guillam et al., 1997; Bady et al., 2006). In this line, it has been recently reported the generation of a GLUT2 sugar detection-deficient (GLUT2-SDD) transgenic line in mice that displays hypoinsulinemia, altered glucose homeostasis, delayed development and loss of glucose in the urine (Stolarczyk et al., 2007).

To date, in fish there are no studies using such approaches due to technical limitations in the generation of germline mutations. Thereby, in our study we have benefited from the zebrafish model and its advantages for the study of gene function *in vivo.* Thus, to study its function during the early developmental stages in zebrafish we

knocked down GLUT2 using antisense morpholinos. Our results showed that embryos lacking GLUT2 display a delay of the whole body development with severe alterations in the midbrain and hindbrain ventricles. We confirmed the structural alteration in the CNS structure by immunostaining of acetylated tubulin. In addition, using marker genes for neural progenitor cells, we found that GLUT2 abrogation may be causing the observed neurodegenerative phenotype as a consequence of alterations in the development of these cells but by mainly affecting the progenitors of the GABAergic neurons.

Next, by studying the functional alterations triggered by the lack of GLUT2 we observed that morphant embryos displayed an impairment of glucose uptake in the whole body but especially in the head region. Interestingly, a similar pattern was found when assaying cell viability in these embryos, showing a significant increase in apoptotic cell death, mainly located in the cephalic area. These evidences led us to hypothesize that insufficient supply of glucose at these critical stages may result in increased apoptotic cell death. Furthermore, blocking the expression of GLUT2 resulted in alterations in the asymmetric distribution of some endoderm-derived organs shown to express this glucose transporter, namely the liver and the endocrine and exocrine pancreas. Using marker genes for these organs, we were able to determine the significance of this effect that caused alterations on the asymmetry axis in the liver in 69% of the morphant embryos, in the exocrine pancreas in 73% of the morphant embryos and in the endocrine pancreas in 55% of the morphant embryos.

Additionally, by studying the transcriptional alterations in these embryos by microarray analysis, we were able to validate the knockdown of GLUT2 since the mRNA expression levels of this gene were strongly down-regulated in morphant embryos. Furthermore, a number of genes involved in apoptotic processes were differentially expressed in morphant embryos, confirming the observed increase in apoptotic cell death. Interestingly we also found that the expression of parvalvumin and PDX1 appeared altered. These genes are marker genes for the GABAergic neurons and the endocrine pancreas, respectively, where they play an important role in their normal functioning (MacFarlane et al., 1994; Marshak et al., 1996; Hashimoto et al., 2003). We

also observed that LEFTY2 and TGFβ1 resulted differentially expressed in morphant embryos as a consequence of the knockdown of GLUT2. Since both genes are important modulators of the Nodal pathway and are required for the normal asymmetric organization of the dorsal diencephalon and endoderm derived tissues (Bisgrove et al., 1999; Rodaway et al., 1999; Bisgrove et al., 2000), these evidences support the observed alterations in the morphant patterning.

To study the functionality of zebrafish GLUT2 using a mammalian in vitro model and to investigate its ability to rescue GLUT2-knocked down pancreatic cells.

In mammals, GLUT2 expression in the liver, intestine, kidney and endocrine pancreas is regulated by the variations in blood carbohydrate levels produced by fasting and re-feeding states (Thorens, 1996). Particularly, in pancreatic β-cells, a rise in circulating glucose results in an increase in glucose uptake mediated by GLUT2, leading to the depolarization of the plasma membrane that the secretion of insulin granules (Deeney et al., 2000; Henquin et al., 2003; Thorens, 2011). The use of transgenic models has been helpful in the study of the physiological role of GLUT2 and its implication in the glucose-stimulated insulin secretion in mammals. Hence, GLUT2 null and SDD transgenic mice appeared to be hyperglycemic and hypoinsulinemic, having lost the glucose-sensing capability necessary for insulin secretion (Guillam et al., 1997; Thorens et al., 2000; Stolarczyk et al., 2007). Interestingly, Guillam et al. showed that re-expression of GLUT2 was able to restore the insulin secretion induced by glucose (Guillam et al., 2000), further demonstrating the importance of this glucose transporter in glucose metabolism.

Thus, we set out to establish an *in vitro* system using the mammalian β-cell line MIN6 (Miyazaki et al., 1990) in order to further study the physiological function of zebrafish GLUT2. Our results showed that we were able to knockdown endogenous GLUT2 leading to a loss of glucose-stimulated insulin secretion in MIN6 cells. Furthermore, we successfully established the conditions for the expression of zebrafish GLUT2 in MIN6 and observed a significantly increase in the basal glucose uptake in the

pancreatic cells. In addition, preliminary results point to a possible increase in the glucose uptake in cells expressing the rat GLUT2 construct.

Therefore, these results constitute the first step on the establishment of a promising in vitro system with which future experiments rescuing the GLUT2 knocked down MIN6 cells with exogenous (zebrafish) GLUT2 will help us better understand the functional properties of zebrafish GLUT2. Moreover, taking advantage of the GFP proteins fused to the transfected constructs, we will be able to study the trafficking properties of zebrafish GLUT2.

Conclusions

1. We have cloned and characterized the first functional GLUT4 promoter in a non-mammalian species. From a structural point of view, this promoter sequence is highly conserved amongst teleost fish and presents the majority of the binding motifs known to be important for the regulation of GLUT4.

2. The Fugu GLUT4 promoter is regulated by insulin, PPARγ and electrical pulse stimulation in mammalian skeletal muscle cells. Stimulation with insulin resulted in a significant reduction of the activity of Fugu GLUT4 promoter. However, GLUT4 promoter activity was increased by PPARγ activation and experimentally controlled contraction.

3- In zebrafish embryos at 24 hpf and 48 hpf, GLUT2 mRNA is detected in the brain and proneprhic ducts. By 72 and 120 hpf, an overall increase of GLUT2 expression is observed in intestine, liver, pancreas, pronephric tubules and hindbrain region.

4- Knockdown of zebrafish GLUT2 *in vivo* causes severe abnormalities in brain organogenesis, resulting in defective formation of the mid-brain/hindbrain boundary and hindbrain, as well as a delay in whole body development.

5- GLUT2 in zebrafish embryos plays a pivotal role during the development of neuronal progenitor cells, especially for the generation of the GABAergic neurons. This suggests the existence of a glucose-sensing region in the zebrafish hindbrain in which these neurons may be involved in the regulation of glucose homeostasis.

6- GLUT2 deficient embryos showed impaired glucose uptake, a generalized increase in apoptotic cell death primarily localized in the head region and alterations in the distribution of endoderm-derived tissues. Therefore, these evidences suggest that the defective coordination between glucose uptake and the demand for carbohydrates during early embryonic stages could be affecting the development of GLUT2-expressing organs and cell viability.

7- Knockdown of endogenous GLUT2 in MIN6 cells led to a loss of glucose-dependent insulin secretion and overexpression of zebrafish GLUT2 in wild type MIN6 resulted in a significant increase in glucose uptake. These evidences confirmed that MIN6 cells are suitable for the study of the functional role of zebrafish GLUT2 in pancreatic β-cells.

Resumen del trabajo

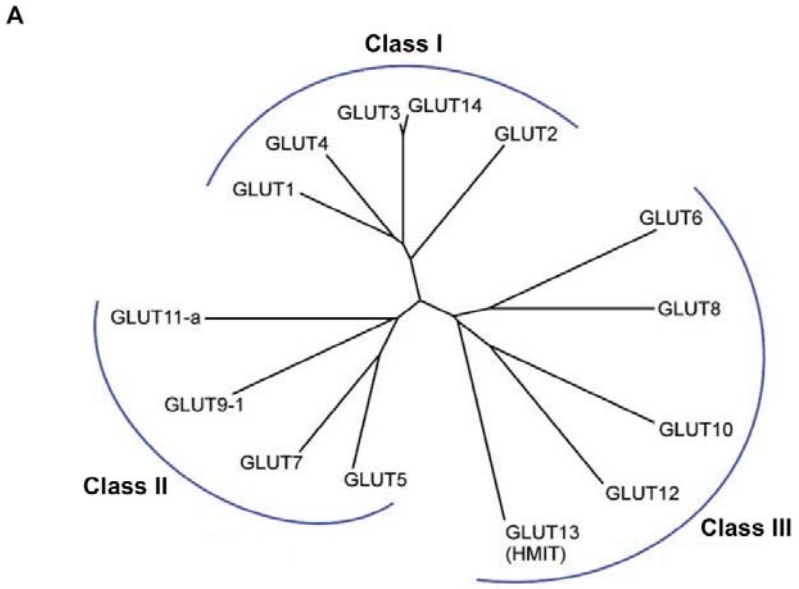
Introducción

1. Transportadores de glucosa

La glucosa es una molécula esencial para el metabolismo de los vertebrados, y desempeña un papel fundamental como combustible y sustrato metabólico (Wood and Trayhurn, 2003). La glucosa se obtiene principalmente de dos maneras: directamente de la dieta y la derivada del glucógeno, que se almacena principalmente en el hígado (Yamashita et al., 2001; Wood and Trayhurn, 2003; Postic et al., 2007). La que es obtenida de la dieta es transferida desde el lumen del intestino delgado a la circulación, y posteriormente dentro de las células objetivo para ser usada como una fuente de energía o, cuando se encuentra en exceso, para ser almacenada como glicógeno hepático o como triglicéridos (Yamashita et al., 2001; Wood and Trayhurn, 2003; Postic et al., 2007). El paso de la glucosa a través de la membrana plasmática esta mediado por dos grupos diferentes de transportadores: los co-transportadores de glucosa Na^+-dependientes (SGLT, miembros de la extensa familia de transportadores Na^+-dependientes, genes de denominados SLC5A) (Wright, 2001); y por los transportadores facilitados de azúcares Na+-independientes (la familia de los GLUT, genes denominados SLC2A) (Mueckler, 1994; Uldry and Thorens, 2004).

La familia de los GLUT pertenece a la principal superfamilia de transportadores facilitados de membrana (MFS) (Pao et al., 1998), siendo la mayor parte de ellos capaces de catalizar la transferencia bidireccional facilitada a través de las membranas plasmáticas (Thorens and Mueckler, 2010). Los GLUTs son proteínas compuestas aproximadamente de 500 aminoácidos que tienen 12 hélices alfa transmembrana y un único N-oligosacárido unido (Joost and Thorens, 2001; Joost et al., 2002; Zhao and Keating, 2007; Augustin, 2010). Los miembros de la familia de los GLUTs pueden ser agrupados en tres clases en función de la similaridad de sus secuencias (Fig. 1). La clase I esta compuesta por los transportadores de glucosa comprendidos entre GLUT1 y GLUT4 y el GLUT14, el cual es una duplicación de GLUT3; la clase II esta compuesta por GLUT5, GLUT7, GLUT9, y GLUT11 y la clase III la integran GLUT6, GLUT8,

GLUT10, GLUT12 y GLUT13 (transportador de mio-inositol acoplado a protones, HMIT) (Joost and Thorens, 2001; Joost et al., 2002; Zhao and Keating, 2007; Augustin, 2010).



B

	Protein	Expression
Class I	GLUT1	Placenta, brain, kidney, colon, eye, skeletal muscle, peripheral nerve and erythrocytes
	GLUT2	Liver, pancreas, small intestine, kidney and brain
	GLUT3	Brain, placenta and testis
	GLUT4	Skeletal muscle, heart and adipose tissue
	GLUT14	Testis
Class II	GLUT5	Testis, intestine, kidney and muscle
	GLUT7	Intestine, testis and prostate
	GLUT9	Liver and kidney
	GLUT11	Pancreas, kidney, placenta and muscle
Class III	GLUT6	Brain, spleen and peripheral leukocytes
	GLUT8	Testis
	GLUT10	Liver and pancreas
	GLUT12	Heart and prostate
	GLUT13	Brain

Figura 1. Los miembros de la familia de transportadores de glucosa. A, Árbol filogenético radial enseñando la relación entre los 14 GLUTs humanos. Adaptado de (Augustin, 2010). **B**, Esquema de las proteínas GLUT indicando el tejido donde cada miembro aparece expresado.

2. Transportadores facilitados de glucosa Clase I

La clase I de transportadores facilitados de glucosa incluye de GLUT1 a GLUT4 y GLUT14, y han sido extensamente caracterizados en términos de estructura, función y distribución tisular (Fig. 1 y 2). GLUT1 se expresa principalmente en la placenta, cerebro (incluyendo la barrera hemato-cerebral), riñón, colon, ojo, músculo esquelético, sistema nervioso periférico y eritrocitos (Joost et al., 2002; Pereira and Lancha, 2004; Zhao and Keating, 2007). GLUT1 juega un papel relevante proporcionando glucosa a las células del sistema nervioso central (Thorens and Mueckler, 2010). GLUT2 se expresa principalmente en células β pancreáticas, donde participa en el mecanismo gluco-sensor, el hígado, intestino, los riñones y en el cerebro (Thorens et al., 1988; Guillam et al., 2000; Joost and Thorens, 2001; Panserat et al., 2001). GLUT2 se expresa en la membrana baso-lateral de los hepatocitos, de los enterocitos y en los túbulos renales proximales permitiendo el transporte bidireccional de glucosa controlado hormonalmente (Thorens et al., 1990; Thorens, 1992; Wright et al., 2003) y se ha visto que es capaz de translocarse a la membrana apical desde el citosol en enterocitos y en los túbulos renales proximales (Kellett and Helliwell, 2000; Marks et al., 2003). GLUT3 tiene una elevada afinidad por la glucosa y esto concuerda con su presencia en tejidos donde la demanda de glucosa como combustible es considerable, principalmente en el cerebro, los testículos y la placenta (Shepherd et al., 1992; Haber et al., 1993; McCall et al., 1994).

El transportador de glucosa sensible a insulina GLUT4, junto con GLUT1, es el transportador de glucosa más intensamente estudiado. Se encuentra expresado en corazón, músculo esquelético y tejido adiposo (Zorzano et al., 1997; Thai et al., 1998; Khayat et al., 2002; Abel, 2004; Watson et al., 2004; Valverde et al., 2005), donde es responsable de paliar el aumento de los niveles de glucosa plasmáticos en la fase postpandrial (Huang and Czech, 2007). La insulina actúa estimulando la translocación de vesículas contenedoras de GLUT4 almacenadas intracelularmente hacia la membrana plasmática, resultando en un incremento inmediato del transporte de glucosa (Bryant et al., 2002). Varios modelos de estudio animal y humano para la

investigación de estados de resistencia a insulina, como la obesidad y la diabetes tipo II, presentan una disrupción en el mecanismo de regulación de GLUT4, lo que sugiere el posible papel clave que juega GLUT4 en patologías de resistencia a la insulina (Huang and Czech, 2007). Por último, GLUT14 se encuentra específicamente expresado en testículo (Wu and Freeze, 2002) y presenta un elevado grado de similitud con GLUT3 (95% a nivel nucleotídico) lo cual, junto a su localización cromosómica, llevó a Wu y colaboradores a proponer que GLUT14 es una duplicación génica de GLUT3 (Wu and Freeze, 2002). Sin embargo, hasta la fecha el papel concreto que desempeña GLUT14 en el metabolismo de la glucosa es desconocido.

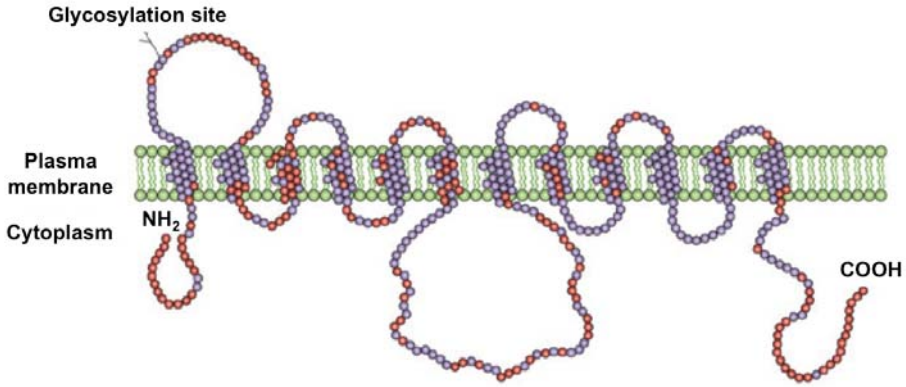


Figura 2. Modelo esquemático de los miembros de la clase I. El diagrama muestra las 12 hélices alfa transmembrana, con el lugar de glicosilación localizado en el primer bucle extracelular y los extremos amino- y carboxilo-terminales situados intracelularmente. Adaptado de (Bryant et al., 2002).

3. GLUT2 (SLC2A2)

GLUT2 fue clonado por primera vez en 1988 a partir de librerías de ADNc de hígado y riñón humano, por Thorens et al. (Thorens et al., 1988). En primer lugar se encontró expresión de GLUT2 en páncreas, hígado, intestino y riñón (Augustin, 2010; Thorens and Mueckler, 2010) y estudios posteriores revelaron expresión también en el cerebro (Leloup et al., 1994; Li et al., 2003). GLUT2 es un transportador de glucosa de baja afinidad y alta capacidad con la *Km* muy elevada (17 mM) (Johnson et al., 1990), la

mayor entre los miembros conocidos de la familia de los GLUTs (Johnson et al., 1990). Como transportador de glucosa de clase I, se ha predicho que GLUT2 tiene la estructura consenso característica de esta clase (Fig. 2) con la única excepción de que el motivo QLS en la hélice 7 no está presente (Burant et al., 1991; Baldwin, 1993). Este motivo es conocido por conferir la especificidad al substrato, y su ausencia podría explicar la elevada afinidad por la D-glucosa, D-fructosa y glucosamina (Burant et al., 1991; Baldwin, 1993; Uldry et al., 2002).

En mamíferos, GLUT2 se expresa en un alto grado en células β pancreáticas, siendo más abundante en las microvellosidades que en la membrana baso-lateral (Orci et al., 1989). GLUT2 se ha encontrado también internalizado en los endosomas tempranos y lisosomas de células β pancreáticas en dos líneas de ratones genéticamente modificados, afectando su capacidad de glicosilación, bloqueando la N-acetilglucosamin transferasa GnT-4a (Ohtsubo et al., 2005); o induciendo la apoptosis en células β pancreáticas activando la caspasa 8 (Wang et al., 2008). GLUT2 también se expresa en las membranas baso-lateral y apical de células epiteliales del intestino y los riñones (Thorens et al., 1990; Thorens, 1992; Kellett and Helliwell, 2000; Marks et al., 2003; Wright et al., 2003), así como en la membrana plasmática sinusoide y en fracciones endosomales de los hepatocitos (Thorens et al., 1990; Thorens, 1992; Eisenberg et al., 2005).

Desde el punto de vista fisiológico, el patrón de expresión de GLUT2, junto con su capacidad de transportar diferentes tipos de hexosas dentro de un amplio rango de concentraciones, asegura un rápido equilibrado de glucosa entre los espacios intra- y extracelulares, permitiendo de este modo flujos bidireccionales desde dentro y fuera de las células (Leturque et al., 2005; Thorens and Mueckler, 2010). Este mecanismo contribuye a un elevado número de procesos (Fig. 3), como la absorción intestinal y renal de glucosa, la estimulación de la secreción de insulina por las células β pancreáticas, la entrada y salida de glucosa del hígado y la capacidad gluco-sensora de regiones específicas del cerebro involucradas en la regulación del metabolismo de la

glucosa y de la toma de alimentos (Burcelin et al., 2000; Guillam et al., 2000; Uldry and Thorens, 2004; Leturque et al., 2005; Marty et al., 2007; Eny et al., 2008).

Concretamente, en células β pancreáticas, la expresión de GLUT2 en la superficie celular es la responsable de la toma de glucosa que desencadenará la secreción de insulina inducida por glucosa (Ohtsubo et al., 2005). Esto provoca la despolarización de la membrana plasmática por el cierre de los canales de K^+ ATP dependientes y el influjo de Ca^{2+}, llevando a la exocitosis de gránulos de insulina (Henquin et al., 2003). En células β pancreáticas, GLUT2 equilibra la glucosa extra- e intracelular proporcionando un suministro de glucosa sin restricciones, siendo la glucokinasa el paso limitante (Matschinsky, 2002). A pesar de esto , se ha descrito que en células β pancreáticas generadas con diferentes isoformas de GLUTs, solo GLUT2 permite la producción normal de insulina en respuesta a la estimulación por glucosa (Hughes et al., 1993).

En enterocitos, esta bien establecido que GLUT2 transloca a la membrana apical desde vesículas localizadas en el citosol tras comidas ricas en azucares (Gouyon et al., 2003; Kellett and Brot-Laroche, 2005). En respuesta al incremento de los niveles plasmáticos de azucares, la insulina es secretada por las células β pancreáticas, haciendo que GLUT2 se internalice (Tobin et al., 2008). De este modo, la capacidad para transportar azúcares es regulada por GLUT2, ajustándose en función de la concentración luminal de los niveles de glucosa (Kellett et al., 2008). De modo similar ocurre en las células del túbulo proximal del riñón de ratas tratados con estreptozotocina, donde GLUT2 es translocado a la membrana de borde en cepillo en respuesta a la glucosa y es internalizado tras el ayuno (Marks et al., 2003).

Curiosamente, GLUT2 también se internalizar en el hígado. En los hepatocitos GLUT2 se expresa en la membrana sinusoide (Thorens et al., 1990), siendo sometido a internalización dependiente de insulina en fracciones endosomales (Eisenberg et al., 2005). De esta manera, este mecanismo podría ser relevante mediando el efecto inhibidor de la insulina sobre la producción hepática de glucosa (Girard, 2006).

Respecto a la expresión de GLUT2 en el cerebro, se sabe poco sobre su función fisiológica. Estudios previos muestran que GLUT2 se encuentra en el cerebelo, el núcleo cerebral, núcleo hipotalámico, neuronas, células de la glía y astrocitos (Leloup et al., 1994; Nualart et al., 1999; Arluison et al., 2004; Arluison et al., 2004; Kang et al., 2004; Roncero et al., 2004; Marty et al., 2005). GLUT2 está implicado en la detección de la hipoglicemia en células de la glía (Marty et al., 2005) y también podría estarlo en la respuesta de contra-regulación mediada por las unidades sensibles a glucosa dependientes de GLUT2 de los astrocitos y neuronas (Marty et al., 2005). Estas evidencias apoyan la idea de que GLUT2 juega un papel central en la regulación de la ingesta, tal y como sugieren varios estudios donde se muestra una conducta alimentaria anormal bloqueando GLUT2 intra-cerebro-ventricularmente en ratas (Wan et al., 1998) o en ratones carentes de GLUT2 (Bady et al., 2006).

En vertebrados nos mamíferos GLUT2 ha sido caracterizado en aves y peces (Wang et al., 1994; Krasnov et al., 2001; Hall et al., 2006; Castillo et al., 2009; Terova et al., 2009). En particular, estudios en peces teleósteos muestran que GLUT2 se expresa en páncreas, hígado, rombencéfalo e hipotálamo en trucha arco iris (*Oncorhynchus mykiss*) en hígado, intestino y riñón en bacalao Atlántico (*Gadus morhua*) y en cerebro, corazón, hígado, riñón, músculo e intestino en lubina (*Dicentrarchus labrax*) (Panserat et al., 2001; Hall et al., 2006; Polakof et al., 2007; Terova et al., 2009); mientras que en pez cebra (*Danio rerio*) adulto GLUT2 se encuentra expresado en un elevado número de tejidos (Fig. 4) (Castillo et al., 2009). La afinidad del GLUT2 de pez cebra por la 2-deoxiglucosa (2-DG) es 11 mM, similar a la constante de mamíferos (Castillo et al., 2009). Además, el GLUT2 de pez cebra también es capaz de transportar manosa, fructosa y galactosa, así como L- glucosa pero con menor afinidad, tal y como se ha visto en mamíferos (Cheeseman, 2002). Estas propiedades indican un elevado grado de conservación funcional entre el GLUT2 de peces y de mamíferos, apoyando la idea de que GLUT2 es un transportador de glucosa de baja afinidad y alta capacidad (Uldry and Thorens, 2004).

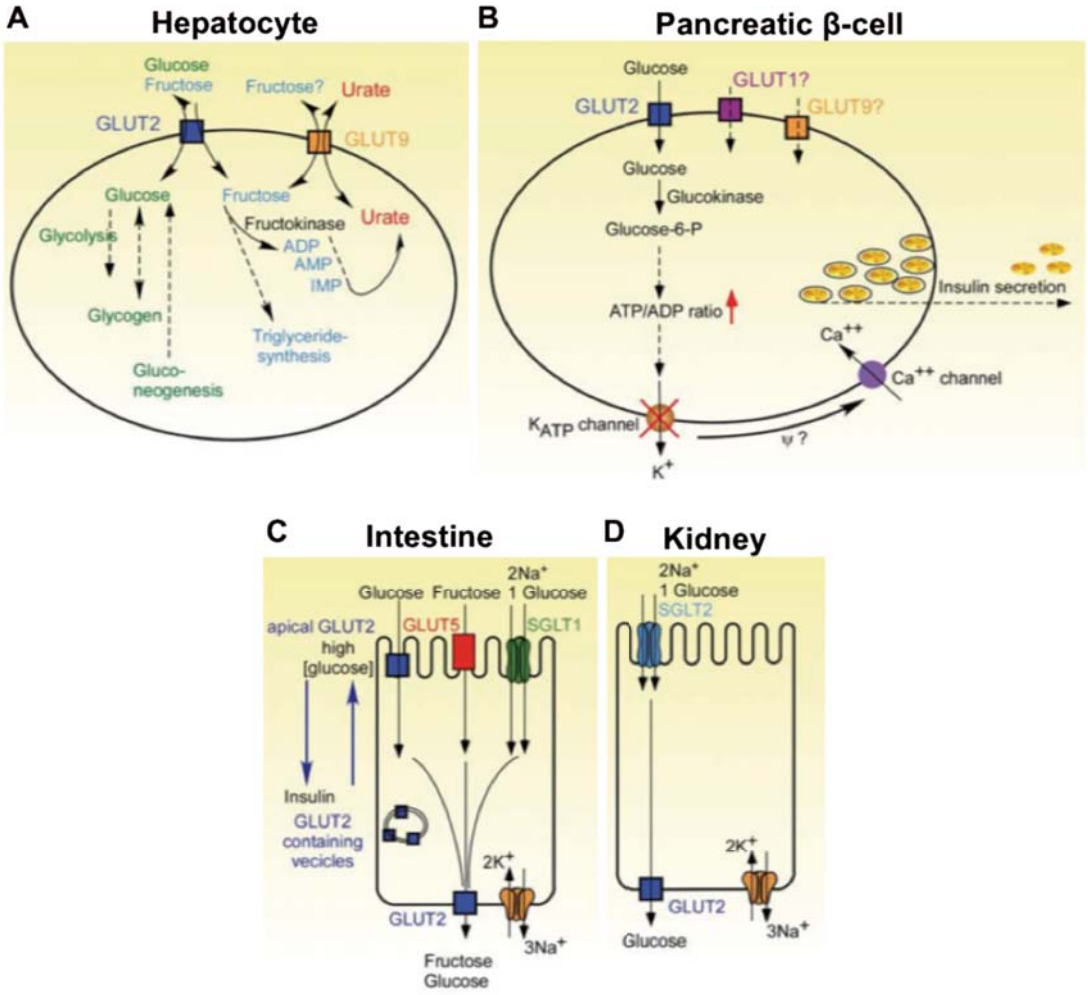


Figura 3. Papel fisiológico de GLUT2 en diferentes órganos. A, Transporte de hexosas en hepatocitos. GLUT2 es el mediador de la captación de glucosa bajo condiciones de alimentación en hepatocitos donde la glucosa se metaboliza por glicólisis o es incorporada a glicógeno. **B**, Las células β pancreáticas secretan insulina en respuesta a aumentos de la glucosa en sangre. GLUT2 interviene en la captación de glucosa en las células β pancreáticas provocando la secreción de insulina. **C**, Absorción transepitelial de glucosa en el intestino. GLUT2 es translocado a la membrana apical epitelial para facilitar la captación de glucosa, dicha translocación es revertida por la acción de la insulina. **D**, Reabsorción transepitelial de glucosa en el riñón. La reabsorción de la glucosa mediada por GLUT2 se da en la membrana baso-lateral. Adaptado de (Augustin, 2010).

Respecto al papel fisiológico de GLUT2 en peces, se sabe poco hasta la fecha. Se ha visto que en teleósteos la expresión de GLUT2 en el páncreas y en el rombencéfalo es regulada por señales hormonales y metabólicas (Polakof et al., 2007), mientras que los niveles de ARNm de GLUT2 en el hígado no están afectados en condiciones de ayuno y realimentación (Panserat et al., 2001; Hall et al., 2006; Castillo et al., 2009). Además, los niveles de ARNm del GLUT2 de peces en el hígado esta sobre-regulado en condiciones de hipoxia aguda y crónica (Terova et al., 2009). Asimismo, estudios recientes han demostrado que la expresión de GLUT2 en pez cebra se encuentra bajo regulación nutricional en el intestino (Castillo et al., 2009).

En vista de esto, resulta evidente que son necesarios más estudios para entender el papel fisiológico de GLUT2 y los mecanismos involucrados en su regulación. Los vertebrados inferiores han sido ampliamente reconocidos como una herramienta valiosa en la investigación metabólica (Schlegel and Stainier, 2007). Además, existe una conservación remarcable en lo que se refiere a estructura y propiedades funcionales de GLUT2 entre peces y mamíferos. En este aspecto, estudios futuros sobre el papel fisiológico de GLUT2 usando una especie modelo, como el pez cebra, nos proporcionara una valiosa contribución para poder desentramar los complejos mecanismos involucrados en la homeostasis de la glucosa.

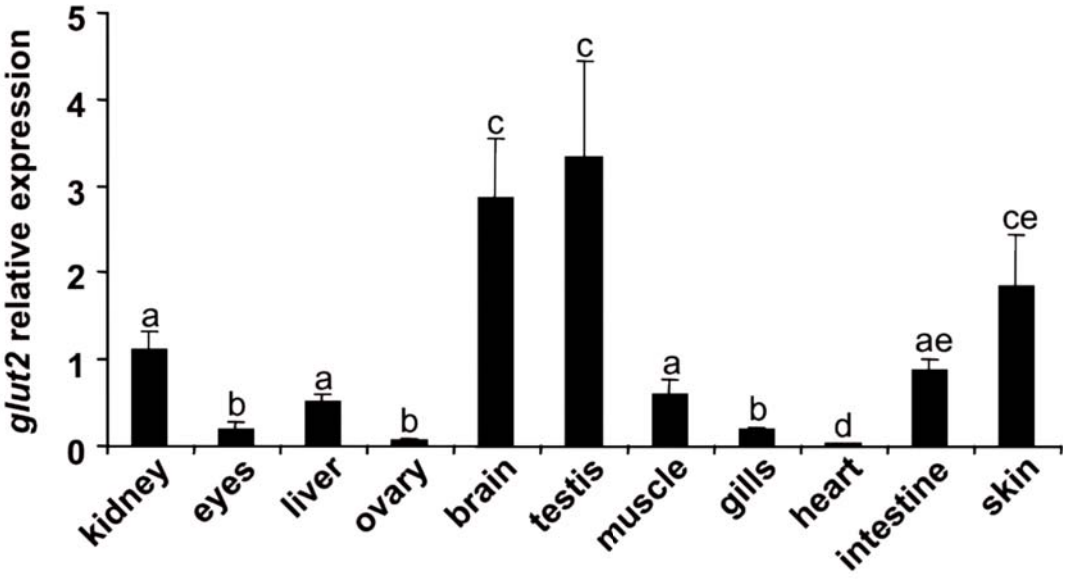


Figura 4. Patrón de expresión tisular de GLUT2 en pez cebra adulto. GLUT2 se expresa en una amplia variedad de tejidos en pez cebra adulto, pero especialmente en riñón, hígado, cerebro, testículo, músculo, intestino y piel. Adaptado de (Castillo et al., 2009).

4. GLUT4 (SLC2A4)

GLUT4 fue descrito por primera vez por James et al. en 1988 estudiando el metabolismo de la glucosa en tejidos regulados por insulina (James et al., 1988). Pronto, tras este descubrimiento varios grupos clonaron GLUT4 en humano (Fukumoto et al., 1989), rata (Birnbaum, 1989; Charron et al., 1989) y ratón (Kaestner et al., 1989). Desde su descubrimiento, GLUT4 ha recibido, junto con GLUT1, más atención científica que cualquier otra proteína transportadora de membrana. Estructuralmente, GLUT4 sigue el modelo predicho para los transportadores de clase I (Fig. 2). Además presenta una gran afinidad por la glucosa, con una *Km* de ≈5 mM (Huang and Czech, 2007), y también es capaz de transportar manosa, galactosa, ácido dehidroascórbico y glucosamina (Keller et al., 1989; Burant and Bell, 1992; Rumsey et al., 2000; Uldry et al., 2002).

En mamíferos, GLUT4 esta principalmente expresado en músculo cardiaco y esquelético, tejido adiposo marrón y blanco, y en cerebro (Mueckler, 1994; Rayner et al., 1994; Huang and Czech, 2007). GLUT4 juega un papel relevante en la homeostasis de la glucosa en todo el cuerpo, mediando la captación de glucosa regulada por insulina (Shepherd and Kahn, 1999; Saltiel and Pessin, 2002). La disrupción de la expresión de GLUT4 ha sido extensamente asociada con patologías con la captación de glucosa afectada y de resistencia a insulina como la diabetes tipo II y la obesidad (Kusari et al., 1991; Shepherd and Kahn, 1999; Friedel et al., 2002; Karnieli and Armoni, 2008).

El gen de GLUT4 se encuentra regulado por mecanismos complejos, ya que esta sujeto tanto a regulación tisular específica como hormonal (Knight et al., 2003). Así, la expresión de GLUT4 está alterada en músculo cardiaco y esquelético en el hipotiroidismo perinatal (Castello et al., 1994; Ramos et al., 2001). Está deficiencia puede ser revertida al tratarla con triyodotironina (T_3), la cual incrementa los niveles de ARNm de GLUT4 en el músculo cardiaco (Castello et al., 1994). Así mismo, administraciones de T_3 a largo plazo en ratas adultas, estimulan la expresión de GLUT4 en músculo esquelético (Casla et al., 1990; Weinstein et al., 1994). La actividad contráctil y la inervación también regulan la expresión de GLUT4 en músculo esquelético. Varios estudios evidencian que la expresión de GLUT4 se induce durante el tiempo de inervación del músculo esquelético (Castello et al., 1993) y es reprimida en respuesta a la denervación muscular (Block et al., 1991; Coderre et al., 1992; Castello et al., 1993). La contracción muscular aumenta la expresión de GLUT4 en músculo esquelético. Diversos estudios demuestran que la expresión de GLUT4 se induce al estimular eléctricamente la contracción muscular *in vivo* e *in vitro* (Etgen et al., 1993; Hofmann and Pette, 1994; Kong et al., 1994) así como tras entrenamiento físico en humanos (Houmard et al., 1991; Dela et al., 1993) y ratas (Ploug et al., 1990).

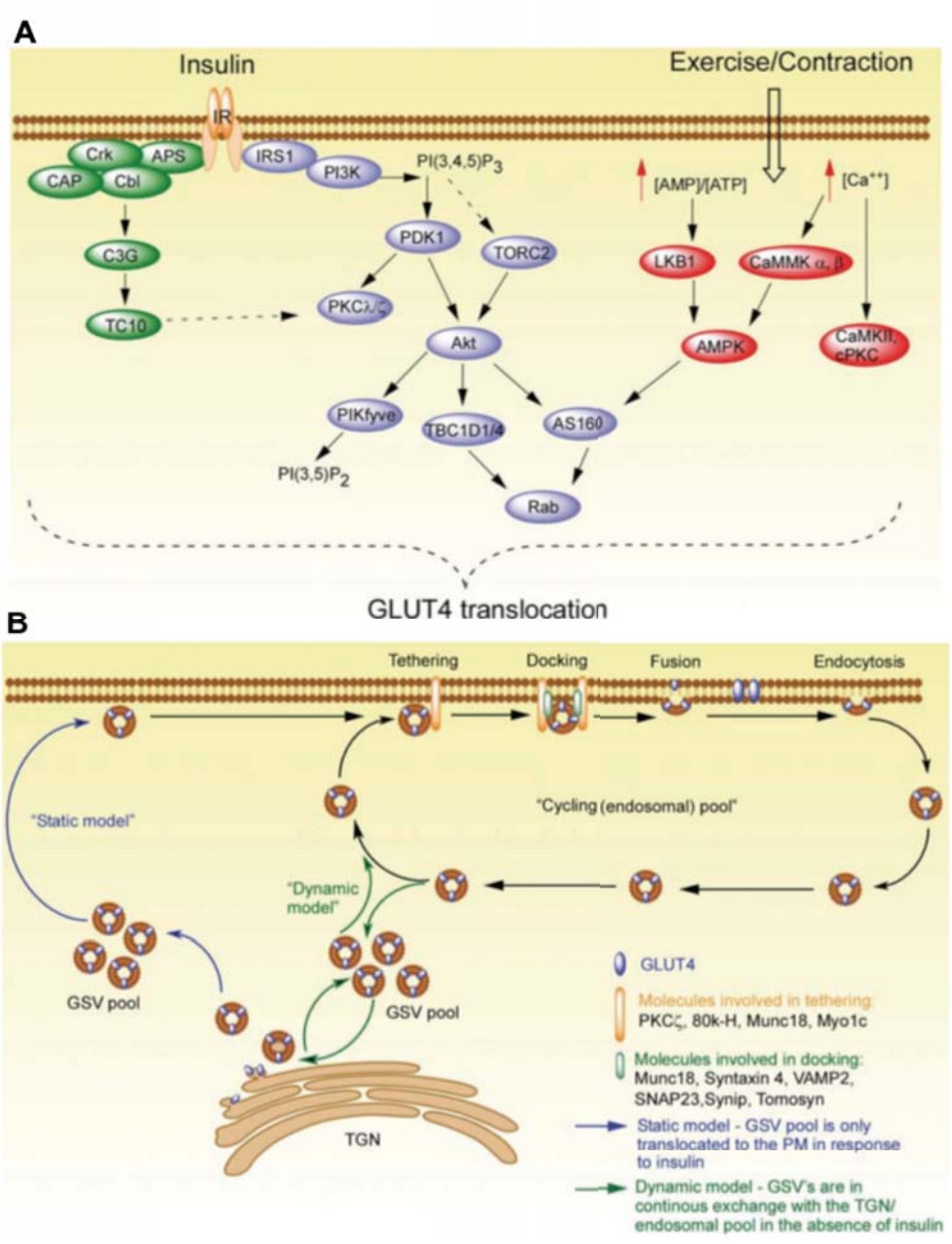


Figura 5. Representación esquemática del mecanismo de regulación de GLUT4. A, Principales vías de señalización involucradas en la translocación de GLUT4. **B**, Vías de tráfico de GLUT4. Adaptado de (Augustin, 2010).

La circulación de GLUT4 es regulada a nivel de su exocitosis, fusión, endocitosis tránsito inter-endosomal. En músculo y tejido adiposo está principalmente regulado por contracción muscular e insulina (Zorzano et al., 2005; Watson and Pessin, 2006;

Augustin, 2010; Lauritzen and Schertzer, 2010), permitiendo la entrada de glucosa en el interior de las células estimulando la translocación de GLUT4 (Fig. 5B) (Augustin, 2010). La insulina regula el tráfico de GLUT4 vía fosfatidilinositol 3 kinasa (PI3K) o el complejo Cbl-CAP-APS (Fig. 5A) (Ishiki and Klip, 2005) mientras que la contracción muscular regula la translocación de GLUT4 vía la proteín kinasa activada por AMP (AMPK) (Fig. 5A) (Pereira and Lancha, 2004; Zorzano et al., 2005). En ausencia de insulina, el GLUT4 fusionado a la membrana plasmática es endocitado por medio de la vía endosomal temprana y es ubicado en el compartimento de vesículas de almacenamiento de GLUT4 (GSV) (también denominado compartimento especializado sensible a insulina) estando involucrada la red trans-Golgi (TNG) (Dugani and Klip, 2005; Ishiki and Klip, 2005; Larance et al., 2008). GLUT4 se almacena mediante procesos estáticos o dinámicos, de donde, en respuesta a estímulos, se translocará a la membrana plasmática (Fig. 5B) (Dugani and Klip, 2005; Ishiki and Klip, 2005; Larance et al., 2008).

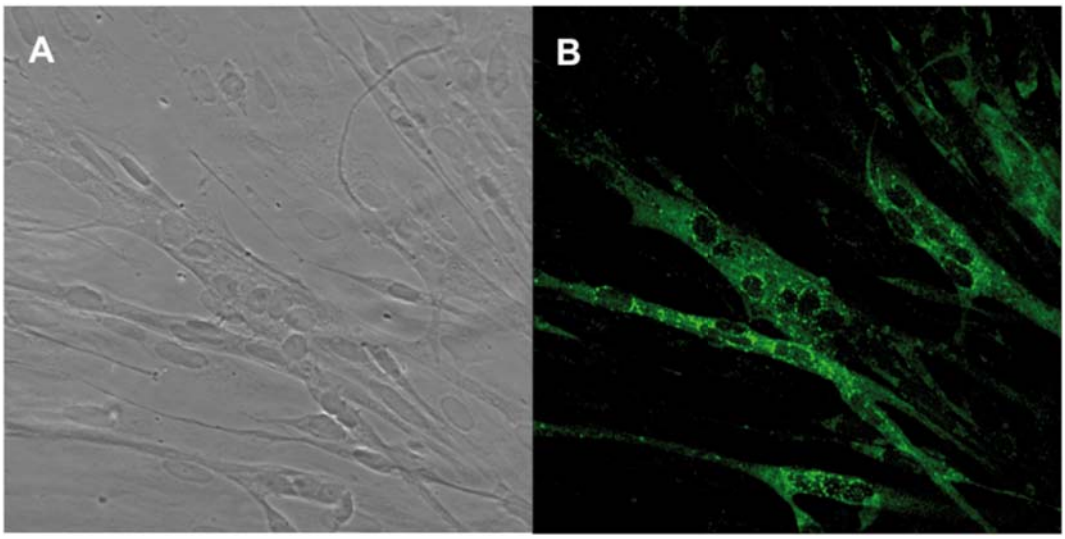


Figura 6. Distribución de GLUT4 en miotubos primarios de trucha marrón. A, Imagen de campo claro de los miotubos de trucha marrón. **B**, Imagen fluorescente enseñando la inmunolocalización del GLUT4 endógeno de trucha marrón. Adaptado de (Diaz et al., 2007).

En vertebrados no mamíferos, GLUT4 fue caracterizado por primera vez por Planas y colaboradores en músculo rojo de trucha marrón (*Salmo trutta*) (Planas et al.,

2000) y, hasta hoy, ha sido también clonado en otras especies de teleósteos (Capilla et al., 2004; Hall et al., 2006). En trucha marrón, GLUT4 se encuentra altamente expresado en músculo rojo (Fig. 6) y blanco, tejido adiposo, riñón y agallas, y más moderadamente en intestino y corazón (Planas et al., 2000). En otras especies de salmónidos, GLUT4 se encuentra también expresado en tejidos sensibles a la insulina como en músculo blanco y rojo de trucha arco iris (Capilla et al., 2002; Diaz et al., 2007; Diaz et al., 2009) y en tejido adiposo de salmón Coho (*Oncorhynchus kisutch*) (Capilla et al., 2004).

Respecto a las características funcionales, como se ha descrito en mamíferos, el GLUT4 del salmón Coho es capaz de transportar glucosa, manosa y galactosa y, curiosamente es capaz de transportar fructosa a elevadas concentraciones (Capilla et al., 2004). Así mismo, el valor de K_m del GLUT4 del salmón Coho para la glucosa es ≈7.6, mayor que en mamíferos (Capilla et al., 2004). Análisis cinéticos adicionales de transporte de 3-*O*-metilglucosa medidos bajo condiciones de intercambio de equilibrio, mostraron un valor de K_m de 14.4 mM del GLUT4 del salmón Coho (Capilla et al., 2004), de nuevo mayores que los valores mostrados en ratas (Keller et al., 1989; Nishimura et al., 1993). Estas evidencias apoyan la idea de que los GLUTs de peces tiene una menor afinidad por la glucosa que los de vertebrados superiores (Capilla et al., 2004) siendo consistente con la reducida capacidad de los peces de utilizar la carga de glucosa comparado con mamíferos (Moon, 2001).

Además, la expresión de GLUT4 en el tejido muscular de peces es regulada por la acción de factores relacionados con el metabolismo de los carbohidratos y de la homeostasis de la glucosa. Los niveles de ARNm de GLUT4 aumentan en respuesta a la administración de insulina en el músculo rojo de trucha marrón (Capilla et al., 2002) y de trucha arco iris (Polakof et al., 2010), y también en mioblastos y miotubos diferenciados *in vitro* (Diaz et al., 2009). En el mismo estudio, Díaz et al. demostraron que el factor de crecimiento insulínico I (IGF-I), estimula la expresión de GLUT4 en cultivos primarios de células de trucha arco iris (Diaz et al., 2009), siendo consistente con datos anteriores en mamíferos (Bilan et al., 1992). En bacalao Atlántico, la

expresión de GLUT4 aumenta en músculo blanco en ayuno y en el corazón se ve reducida, mientras que los niveles de ARNm retornan a niveles pre-ayuno con la realimentación (Hall et al., 2006). Recientemente, un estudio de nuestro grupo ha mostrado que el 5-aminoimidiazol-4-carboximida ribonucleósido (AICAR) y la metformina, activadores de AMPK, aumentan la expresión de GLUT4 en miotubos de trucha marrón (Magnoni et al., 2012).

Así mismo, de la misma manera que con el ARNm, los niveles de proteína de GLUT4 de trucha marrón en músculo rojo, disminuyen significativamente tras el ayuno y aumentan tras la administración de insulina (Diaz et al., 2007). También, en cultivos primarios de células de músculo esquelético de trucha marrón, Díaz et al. demostraron que la translocación de GLUT4 a la membrana plasmática es estimulada en respuesta a insulina (Fig. 7A), resultando en un aumento de la captación de glucosa en dichas células (Fig. 7B) (Diaz et al., 2007). Estas evidencias se encuentran en concordancia con datos previos en células satélite musculares de trucha arco iris (Castillo et al., 2004). Además, estudios usando la línea celular estable L6 expresando GLUT4 marcado con el epítopo myc también han demostrado que el factor de necrosis tumoral alfa (TNFα) y los activadores de AMPK, AICAR y metformina, estimulan significativamente la translocación a la membrana plasmática de GLUT4 en trucha marrón (Vraskou et al., 2011; Magnoni et al., 2012). Estos mismos estudios también mostraron que la estimulación con TNFα y los activadores de AMPK estimulan la captación de glucosa en células de músculo esquelético de trucha (Vraskou et al., 2011; Magnoni et al., 2012).

En lo que se refiere a la regulación de GLUT4 en tejido adiposo en peces, Capilla et al. fueron capaces de determinar que el GLUT4 de trucha marrón y de salmón Coho se transloca a la membrana plasmática en respuesta a la insulina expresándolo en adipocitos 3T3-L1 (Capilla et al., 2004; Capilla et al., 2010). Además, en el mismo estudio los autores confirman que, como ocurre en mamíferos, la captación de glucosa aumenta en oocitos de *Xenopus* que expresaban el homologo de GLUT4 de salmón, al ser estimulados con insulina (Capilla et al., 2004). Estos mismos resultados concuerdan

con la evidencia de que la insulina aumenta significativamente la captación de glucosa en tejido adiposo de trucha (Capilla et al., 2004).

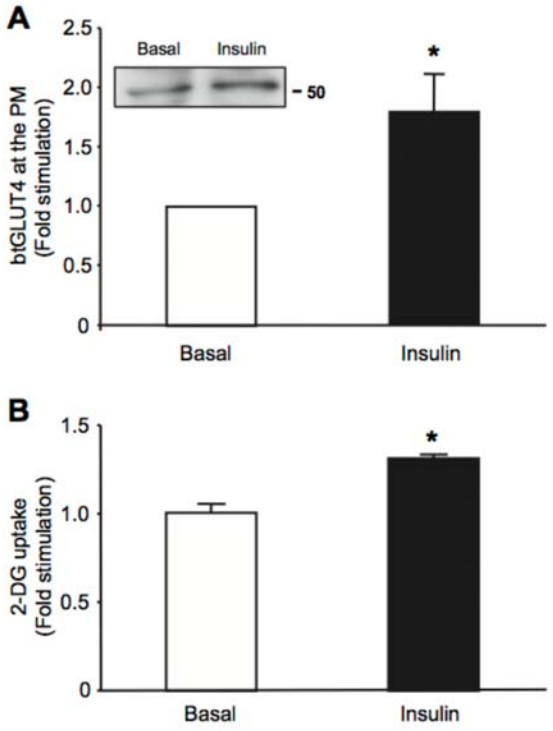


Figura 7. Efectos de la insulina en células musculares de trucha marrón. A, El GLUT4 endógeno de trucha marrón aumenta en membrana plasmática en respuesta a la administración de insulina. **B**, La captación de glucosa aumenta significativamente en las células estimuladas con insulina. Adaptado de (Diaz et al., 2007).

A pesar de estos estudios previos, se sabe poco respecto a los complejos mecanismos que tienen lugar en la regulación y acción de los GLUTs, así como de su papel fisiológico en el metabolismo de peces teleósteos. Concretamente, el modo en que la insulina, una de las principales moléculas implicada en el metabolismo glucídico en mamíferos, regula el metabolismo de la glucosa ha sido poco caracterizado en vertebrados inferiores hasta la fecha.

En vista de la necesidad de aportar más datos para ayudar a descifrar este complejo mecanismo, el objetivo de esta tesis es estudiar los principales transportadores de glucosa involucrados en metabolismo de la glucosa mediado por insulina, GLUT2 y GLUT4. En este aspecto, hemos estudiado, por primera vez, los factores que gobiernan la expresión génica de GLUT4 en un vertebrado inferior, caracterizando los elementos clave de la maquinaria transcripcional en peces usando el pez globo (*Tetraodon nigroviridis*) como especie modelo. Del mismo modo, hemos investigado el papel fisiológico en el metabolismo de la glucosa en embriones de pez cebra así como su importancia durante las fases tempranas del desarrollo. Además, nos hemos propuesto establecer un nuevo sistema *in vitro* para el estudio de la función del GLUT2 de pez cebra usando MIN6-B1, una línea estable de células pancreáticas de ratón.

Resúmenes de los capítulos

Capítulo 1

El transportador de glucosa 4 (GLUT4) juega un papel clave en la captación de glucosa y el metabolismo de los tejidos diana de la insulina. Este transportador ha sido ampliamente estudiado en muchas especies en términos de función, expresión y translocación, pero los estudios sobre la regulación transcripcional se han limitado a ratas, ratones y humanos.

En este estudio se ha clonado y caracterizado un promotor funcional del gen GLUT4 en el Fugu (*Takifugu rubripes*), siendo este el primer promotor no mamífero de GLUT4 que se ha descrito hasta la fecha. Análisis *in silico* del promotor nos ha permitido identificar potenciales sitios de unión tales como SP1, C/EBP, MEF2, KLF, SREBP-1c y cajas GC, así como una isla CpG, pero no se identifico ninguna caja TATA. El análisis *in vitro* reveló tres sitios de inicio de transcripción, donde el principal se encontraría 307 pb aguas arriba del codón ATG. También analizando constructos deleccionados se determinó que el núcleo promotor se encuentra entre los nucleótidos -132 / +93. Mediante la transfección de dichas delecciones 5' hemos podido confirmar que la transcripción del promotor de GLUT4 de Fugu está regulado por la insulina, PG-J2 y estimulación eléctrica. Además, estos experimentos sugieren la implicación de los motivos, como PPAR/RXR y HIF-1a en la regulación por PPARγ y la actividad contráctil, siendo consistente con la literatura de mamíferos. Estos datos confirman que el promotor de GLUT4 de Fugu es un promotor funcional del gen GLUT4 con estructura similar a las descritas en los mamíferos. Además nuestros datos demuestran que este promotor está regulado por mecanismos implicados en la regulación de la expresión de GLUT4 en mamíferos.

Capítulo 2

De entre los 14 miembros de la familia de los transportadores facilitados de glucosa (GLUTs), se ha demostrado en mamíferos que GLUT2 se expresa en una gran variedad de diferentes tejidos, predominantemente en el cerebro, hígado, páncreas, intestino delgado, y el riñón. Además, GLUT2 juega un papel importante en diferentes procesos tales como la absorción intestinal y renal de la glucosa, la estimulación de la secreción de insulina por la glucosa en las células beta del páncreas, la entrada y la salida de la glucosa por el hígado, y la capacidad glucosensora de las regiones específicas del cerebro implicadas en la regulación del metabolismo de la glucosa y la ingesta de alimentos. A pesar de la información disponible en vertebrados superiores, poco se sabe sobre el papel fisiológico de GLUT2 en vertebrados no mamíferos.

De este modo, en base a los resultados previos de nuestro grupo, hemos llevado a cabo estudios para localizar la expresión de GLUT2 y estudiar el papel fisiológico de GLUT2 *in vivo* en un organismo modelo como el pez cebra (*Danio rerio*). En este sentido, hemos localizado la expresión de GLUT2 en el pez cebra durante el desarrollo temprano por hibridación *in situ* (ISH). Para una mejor comprensión de su función in vivo, hemos bloqueado la expresión del GLUT2 de pez cebra mediante la inyección de dos morfolinos diferentes por separado. El primero de ellos inhibiendo el sitio de inicio de traducción (ATG MO) y el segundo diseñado para interferir con la unión entre los exones 5 y 6 (splice MO), que codifican para el dominio de transmembrana 6 (TM6), descrito como importante para la actividad de transporte de glucosa de GLUT2 en los mamíferos.

Nuestros resultados indican que GLUT2 en pez cebra se expresa en el hígado, los túbulos pronéfricos, intestino anterior, páncreas endocrino y las neuronas de la región del cerebelo. Por otra parte, la inactivación de GLUT2 *in vivo* resulta en graves anomalías en la organogénesis del cerebro y afecta a la formación del sistema digestivo, provocando cambios en el eje de asimetría. Curiosamente, los embriones carentes de GLUT2 mostraron alteraciones en absorción de glucosa y una disminución

de la viabilidad celular asociada con aumento significativo en la apoptosis en todo el embrión pero principalmente en la región cefálica. En conjunto, estos resultados establecen la relevancia de GLUT2 en el desarrollo de los órganos principales implicados en el metabolismo de la glucosa posiblemente debido a una baja disponibilidad de carbohidratos, derivando en un incremento de la muerte celular por apoptosis.

Capítulo 3

La insulina juega un papel fundamental la regulación de la homeostasis de la glucosa en los vertebrados superiores, donde la captación de glucosa supone el paso inicial en la secreción de insulina estimulada por glucosa por las células β pancreáticas. En los mamíferos, GLUT2 ha sido descrito como el transportador de glucosa más importante expresado en las células β pancreáticas.

En peces teleosteos se sabe poco respecto al papel que juega GLUT2 en la homeostasis de la glucosa y la acción de la insulina. Es por esto que en este trabajo nos proponemos establecer un sistema *in vitro* usando la línea estable de células β pancreáticas MIN6. Dicha línea deriva de células de insulinoma un ratón transgénico, y presenta secreción de insulina dependiente de glucosa asemejándose a células β pancreáticas normales. En este trabajo, mediante el uso de ARNs cortos de interferencia (siRNAs) específicamente diseñados contra GLUT2, hemos bloqueado la síntesis proteica del transportador de glucosa endógeno de las células MIN6. A continuación, se analizaron las consecuencias funcionales derivadas del bloqueo de la expresión de GLUT2. Para ello se realizaron mediciones de los niveles de insulina secretada por MIN6 GLUT2-deficientes estimuladas con glucosa, confirmando que dichas células perdían su capacidad de secretar insulina en respuesta a glucosa. Por último, hemos realizado ensayos de sobreexpresión mediante la transfección de vectores de expresión que contenían la secuencia codificante completa de los GLUT2 de rata y de pez cebra fusionados a la proteína fluorescente EGFP. Así, observamos que las células MIN6 transfectadas con el GLUT2 de pez cebra mostraban un aumento significativo de la capacidad de transportar glucosa. En resumen, en este estudio se han aportado pruebas que indican que las células MIN6 son adecuadas para el estudio del papel funcional del GLUT2 de pez cebra en las células β pancreáticas. Sin embargo, las condiciones experimentales han de ser optimizadas en trabajos futuros, con el fin de proporcionar pruebas sólidas sobre la funcionalidad del GLUT2 de pez cebra en la secrección de insulina gluco-dependiente.

Discusión

El objetivo de esta tesis fue estudiar la función y regulación de dos de los principales implicados en el metabolismo glucídico regulado por la insulina, los transportadores de glucosa GLUT2 y GLUT4, en los peces teleósteos. En vertebrados no mamíferos, GLUT2 ha sido poco caracterizado hasta la fecha. Se ha demostrado en varias especies de teleósteos que GLUT2 se expresa en los principales tejidos sensibles a la insulina (Krasnov et al., 2001; Hall et al., 2006; Polakof et al., 2007; Castillo et al., 2009; Terova et al., 2009), similar a lo que se describe en los mamíferos (Joost and Thorens, 2001; Uldry and Thorens, 2004). Sin embargo, las propiedades funcionales y el papel fisiológico de GLUT2 apenas ha sido descrito en peces. En vista de ello, se ha caracterizado GLUT2 en el pez cebra, ya que dicha especie es uno de los modelos más reconocidas para el estudio de la fisiología, el desarrollo y el metabolismo (Briggs, 2002; Gerhard, 2003, Schlegel and Stainier, 2007). En cuanto a GLUT4, nuestro grupo ha sido pionero en la investigación de este transportador en peces teleosteos desde que Planas et al. caracterizaron el primer homologo de GLUT4 en vertebrados inferiores (Planas et al., 2000). A pesar de que se ha estudiado más este transportador de glucosa que GLUT2, la información sobre los factores que intervienen en la regulación de la transcripción del gen GLUT4 han sido escasamente caracterizados en mamíferos, mientras que no hay datos disponibles en los vertebrados inferiores . Para ello, en este estudio hemos analizado la regulación de un promotor de GLUT4 de teleósteos bajo la acción de estímulos con conocida capacidad para modular la transcripción y la expresión de GLUT4 en los mamíferos, como la insulina (Cooke and Lane, 1998; Cooke and Lane, 1999; Girón et al., 2008), la contracción de fibras musculares (Slentz et al., 1992; MacLean et al., 2002; Silva et al., 2005; Lima et al., 2009) y PPARs (Armoni et al., 2003). Así, los principales resultados obtenidos a partir de los estudios realizados de acuerdo con los objetivos propuestos se enumeran a continuación:

Estudiar la regulación del gen GLUT4 en los peces teleósteos mediante la identificación y caracterización de una región promotora funcional del gen GLUT4 de Fugu (Takifugu rubripes) y la determinación de las regiones principales importantes para la actividad del promotor

En los mamíferos, GLUT4 juega un papel fundamental regulando la captación de glucosa estimulada por insulina en músculo esquelético y cardíaco y tejido adiposo blanco y marrón (Huang and Czech, 2007). La insulina se ha estudiado ampliamente como el principal modulador de este transportador debido a su implicación directa en patologías con captación de la glucosa alterada y resistencia a la insulina como la diabetes tipo 2 y obesidad (Kusari et al., 1991; Shepherd and Kahn, 1999; Friedel et al., 2002, Karnieli and Armoni, 2008). Sin embargo, GLUT4 también está bajo la regulación u otros factores, ente ellos la contracción muscular, ya que se genera una demanda de glucosa como suministro de energía para las células (Lauritzen and Schertzer, 2010).

También GLUT4 en los peces está regulado por la acción de factores relacionados con el metabolismo de la glucosa y la captación de glucosa estimulada por insulina. Varios estudios describen cómo en los peces se regula GLUT4 en el tejido muscular y adiposo a nivel de expresión de ARN, síntesis de proteínas y translocación a la membrana plasmática (Capilla et al., 2002; Capilla et al., 2004; Hall et al., 2006, Díaz et al., 2007; Díaz et al., 2007; Díaz et al., 2009; Capilla et al., 2010; Polakof et al., 2010; Vraskou et al., 2011). Sin embargo, no hay información respecto a los mecanismos involucrados en la regulación de la transcripción del gen GLUT4 en teleósteos. Con el fin de investigar el papel que juegan los distintos factores que ejercen un control sobre la transcripción del gen GLUT4, se clonó con éxito el promotor de GLUT4 en Fugu. La región 5'-flanqueante del gen de GLUT4 de Fugu mostró características similares a la de mamíferos, como TSS múltiples, ausencia de cajas TATA, regiones ricas en GC cercanas al núcleo del promotor y varios sitios de unión para factores nucleares de transcripción como SP1 y C/EBP (Kaestner et al., 1990; Buse et al., 1992; Liu et al., 1992; Richardson and Pessin, 1993). Estructuralmente, el análisis comparativo entre la secuencia del promotor clonado con promotores de otras especies de peces revelaron

un alto grado de conservación entre dichas especies y que en Fugu el promotor de GLUT4 presenta dos regiones muy conservadas, una de las cuales contiene el casete E-box/MEF2/Klf. Este casete también esta presente en los promotores de mamíferos GLUT4 (Zorzano et al., 2005), donde desempeña un papel importante en la expresión específica de GLUT4 en los tejidos (Liu et al., 1994; Olson and Pessin, 1995; Thai et al., 1998).

Además, se confirmó la funcionalidad del promotor clonado el cual, a su vez, mostró una actividad basal 2-veces mayor que el promotror de GLUT4 de rata. Adicionalmente, mediante la generación de varios constructos conteniendo delecciones del promotor se pudo determinar el promotor mínimo funcional, localizando el núcleo promotor entre los nucleótidos -132 y +93.

Describir los efectos ejercidos en la regulación transcripcional de GLUT4 de Fugu por el agonista de PPARγ, insulina y la contracción en células de músculo esquelético

GLUT4 está regulado a diferentes niveles por un gran numero de factores y, a pesar de la amplia atención experimental que ha recibido, los mecanismos mediante los cuales insulina y otros mediadores ejercen su acción sobre GLUT4 se desconocen (Thorens and Mueckler, 2010). Se sabe que en estados fisiológicos donde la homeostasis de la glucosa se encuentra alterada dan lugar a variaciones en los niveles de ARNm de GLUT4 en el músculo y tejido adiposo. En concreto, se ha descrito que la expresión de este transportador en músculo esquelético aumenta con la contracción muscular provocada por el ejercicio físico y se ve reducida en estados de deficiencia de insulina, incluyendo diabetes tipo II y obesidad. Por lo tanto, el principal objetivo de este trabajo ha sido mejorar nuestra comprensión de los procesos moleculares que regulan la homeostasis de la glucosa por desentrañar los mecanismos que intervienen en la regulación de la transcripción del gen GLUT4.

Como se mencionó antes, la insulina estimula la expresión de GLUT4, la síntesis

de proteínas y su translocación a la superficie celular en los mamíferos (Bryant et al., 2002; Ishiki and Klip, 2005; Larance et al., 2008) y en peces (Capilla et al., 2004 ; Díaz et al., 2007). Sin embargo, el efecto ejercido por esta hormona sobre GLUT4 a nivel transcripcional ha sido poco caracterizado hasta la fecha. En este estudio, hemos observado que la actividad del promotor de GLUT4 de Fugu es inhibida por la insulina de forma dosis y tiempo dependiente, siendo consistentes con las evidencias anteriores en mamíferos (Cooke and Lane, 1998; Cooke and Lane, 1999; Girón et al., 2008). Más aún, todas los constructos conteniendo delecciones fueron reprimidos por la insulina, lo que sugiere la presencia de elementos reguladores aguas abajo del TSS. Estos resultados nos llevan a la hipótesis de que tal vez el promotor de Fugu clonado carece de la región (s) necesaria(s) para la activación transcripcional de GLUT4 por la insulina. En este sentido más estudios serán necesarios para identificar tales regiones.

A continuación, se investigaron los efectos de la activación por PPARγ en células L6 musculares de ratón transfectadas con el promotor clonado de GLUT4 de Fugu. Los PPARs son factores de transcripción activados mediante ligando de la familia de receptores nucleares (Fajas et al, 2001). Entre las tres isoformas de PPAR (α, β y γ), PPARγ se expresa principalmente en los tejidos sensibles a la insulina, como el músculo y el tejido adiposo, jugando un papel importante en el metabolismo lipídico y la regulación de la homeostasis de la glucosa (Fajas et al., 1997; Olefsky and Saltiel, 2000). Por lo tanto, se estudiaron los efectos de la PG-J2, un conocido agonista endógeno de PPARγ (Forman et al., 1995, Kliewer et al., 1995), como un modulador de la transcripción del gen GLUT4 de Fugu. Nuestros resultados mostraron que la estimulación con PG-J2 estimuló significativamente la actividad del promotor de GLUT4 de Fugu y que este efecto desapareció en las delecciones del promotor de GLUT4 de Fugu carentes de los motivos PPAR/RXR. Este resultado concuerda con el hecho de que los receptores PPARγ regulan la transcripción heterodimerizando con RXR (Armoni et al., 2003).

Por último, se investigaron *in vitro* los efectos de la contracción muscular usando la línea de células musculares C2C12 expresando el promotor de GLUT4 de Fugu. El

uso de este sistema para imitar los efectos del ejercicio en miotubos diferenciados *in vitro*, mostró un alto incremento en la actividad transcripcional del promotor de GLUT4 de Fugu. Nuestros resultados también sugieren la importancia del motivo de unión de HIF-1 para la activación transcripcional del promotor, en respuesta a la contracción muscular ya que todas las delecciones que contenían el motivo de HIF-1 aumentaron significativamente su tasa de transcripción en respuesta a la estimulación con impulsos eléctricos, de acuerdo con la literatura en los mamíferos (Silva et al., 2005; Lima et al., 2009). En conjunto, los resultados indican claramente que la contracción muscular estaría regulando la transcripción del gen GLUT4 y sugieren la implicación de HIF-1 como un modulador de la transcripción de GLUT4 en condiciones hipóxicas derivadas del ejercicio físico (Wagner, 2001).

Estudiar la localización de la expresión de GLUT2 en embriones de pez cebra (Danio rerio) a lo largo de las primeras etapas de desarrollo

GLUT2 es el miembro de la familia SLC2 con la capacidad de transportar la mayor cantidad de azúcares de la dieta, sobre todo glucosa, y se expresa en los principales tejidos (hígado, intestino, páncreas endocrino, etc.) que intervienen en la manipulación de los hidratos de carbono (Bell et al., 1990; Thorens and Mueckler, 2010). Debido a su baja afinidad y alta capacidad, este transportador de glucosa permite manejar grandes flujos de azúcares, equilibrando los niveles de glúcidos dentro y fuera de las células. Además, GLUT2 es el principal transportador de glucosa expresado en las células β pancreáticas, mediando la secreción de insulina inducida por glucosa (Henquin et al., 2003). Los estudios previos sobre el GLUT2 de peces, indican que la distribución de tejidos y las propiedades funcionales de este transportador de glucosa son similares a las de los mamíferos (Castillo et al., 2009). Sin embargo, se sabe poco acerca de la función fisiológica de GLUT2 en los vertebrados inferiores. En los peces teleósteos, se sabe que GLUT2 esta regulado por niveles de glucosa plasmática en el cerebro posterior y el páncreas (Polakof et al., 2007) y por la disponibilad de nutrientes en el intestino. Sin embargo, en condiciones de ayuno/re-alimentación los datos de los experimentos son contradictorios a los disponibles en

mamíferos, ya que la expresión de GLUT2 no se altera en el hígado de la trucha arco iris ni del bacalao del Atlántico (Panserat et al., 2001; Hall et al., 2006), mientras que en el pez cebra se encuentra inversamente regulado en el intestino (Castillo et al., 2009). Aparte de las diferencias en la regulación nutricional de la expresión de GLUT2 que se podrían atribuir a las diferencias entre especies, se sabe muy poco respecto a la función de GLUT2 durante el desarrollo temprano. Por esta razón, se estudió la localización de la expresión de GLUT2 durante las primeras fases del desarrollo y los efectos del bloqueo de GLUT2 en los embriones de pez cebra.

El patrón de expresión de GLUT2 durante el desarrollo embrionario del pez cebra se ha estudiado por ISH, observando transcritos en el hígado, los túbulos pronéfricos, intestino anterior, páncreas endocrino y las neuronas que rodean la región del cerebro posterior. La expresión en el sistema pronéfrico y en el cerebro se detectó temprano en el desarrollo, a partir del 24 hpf. El ARNm de GLUT2 se detectó por primera vez en el hígado, páncreas endocrino e intestino anterior a 72 hpf, coincidiendo con la finalización de la formación del aparato digestivo (Wallace and Pack, 2003). También en este punto, la expresión renal de GLUT2 se localiza principalmente en los túbulos pronéfricos y la expresión en el cerebro se localizó principalmente en el cerebro posterior, tanto en el cerebelo y como en el bulbo raquídeo. Estos resultados apoyan la idea de la existencia de una región sensible a la glucosa en el cerebro de pez cebra, tal y como se hipotetizó en trucha arco iris (Polakof et al., 2007) y en mamíferos (Marty et al., 2007).

En resumen, estas evidencias concuerdan con la distribución observada en otros peces teleósteos (Panserat et al., 2001; Hall et al., 2006; Polakof et al., 2007; Castillo et al., 2009; Terova et al., 2009), y en los mamíferos (Thorens et al., 1988; Fukumoto et al., 1989; Bell et al., 1990; Thorens et al., 1990), y sugiere la importancia de GLUT2 para el desarrollo de los tejidos derivados del endodermo y refuerza la hipótesis de la existencia de una unidad de detección de glucosa en el cerebro de pez cebra.

Evaluar los efectos del knockdown de GLUT2 en pez cebra con morfolinos antisentido en el desarrollo embrionario y describir las alteraciones funcionales causadas por la reducción de GLUT2

Varios estudios han recurrido al uso de organismos GLUT2-deficientes para analizar la función de este transportador. Ratones GLUT2-nulos muestran secreción deficiente de insulina, presentando un fenotipo diabético, sobreviviendo solo al ser alimentados con pellets de bajo contenido en azúcar (Guillam et al., 1997). Además, ratones knockout para GLUT2 presentan hiperglucemia, niveles altos de ácidos grasos libres en plasma, glucosuria y comportamiento anormal en la alimentación (Guillam et al., 1997; Bady et al., 2006). En esta línea, recientemente se ha informado de la generación de una línea de ratones transgénicos con detección deficiente de azúcar por GLUT2 (GLUT2-SDD) que muestran hipoinsulinemia, la homeostasis de la glucosa alterada, retraso en el desarrollo y la pérdida de glucosa en la orina (Stolarczyk et al., 2007).

Hasta la fecha, no existen estudios en los peces utilizando tales aproximaciones, debido a limitaciones técnicas. De esta manera, en nuestro estudio nos hemos beneficiado del modelo de pez cebra y sus ventajas para el estudio de la función génica *in vivo*. Así, para estudiar la función durante las primeras etapas del desarrollo del pez cebra se bloqueo GLUT2 utilizando morfolinos antisentido. Nuestros resultados mostraron que los embriones que carecen de GLUT2 presentan un retraso en el desarrollo de todo el cuerpo con alteraciones severas del cerebro medio y los ventrículos cerebrales posteriores. Se confirmó la alteración estructural del sistema nervioso central por inmunolocalización de la tubulina acetilada. Además, mediante el uso de genes marcadores de células progenitoras neurales, se encontró que la abrogación de GLUT2 puede ser la causa de este fenotipo neurodegenerativo como consecuencia de alteraciones en el desarrollo de dichas células pero principalmente afectando a las progenitoras de las neuronas GABAérgicas.

A continuación, mediante el estudio de las alteraciones funcionales provocadas

por la falta de GLUT2 se observó un deterioro de la captación de glucosa en el cuerpo, pero especialmente en la región de la cabeza. Curiosamente, un patrón similar se encontró analizando la viabilidad celular en estos embriones, que mostraron un aumento significativo de la muerte celular por apoptosis, principalmente en la zona cefálica. Estas evidencias nos llevaron a hipotetizar que la disponibilidad insuficiente de glucosa en los momentos críticos del desarrollo puede resultar en un aumento de la muerte celular por apoptosis. Además, el bloqueo de la expresión de GLUT2 resultó en alteraciones en la distribución asimétrica de algunos órganos derivados del endodermo que expresaban el transportador de glucosa, es decir, el hígado y páncreas endocrino y exocrino. Usando genes marcadores para determinar la importancia de este efecto se observaron alteraciones en el eje de la asimetría en el 69% de los casos para el hígado, el 73% para el páncreas exocrino y en el 55% de los casos para el páncreas endocrino.

Además, estudiando las alteraciones transcripcionales de estos embriones mediante análisis por microarray, validamos el knockdown de GLUT2 ya que se encontraba altamente infra-regulado en embriones morphant. Adicionalmente, un número elevado de genes involucrados en los procesos apoptóticos fueron expresadas diferencialmente en los embriones GLUT2-deficientes, confirmando el incremento observado en apoptosis. También curiosamente, se encontró que la expresión de parvalvumina y PDX1 estaba alterada. Estos genes se expresan en las neuronas GABAérgicas y el páncreas endocrino, respectivamente, donde juegan un papel importante en su funcionamiento (MacFarlane et al., 1994; Marshak et al., 1996; Hashimoto et al., 2003). También se observó que LEFTY2 y TGFβ1 resultaron diferencialmente expresados en morphants como una consecuencia de la disminución de GLUT2. Dado que los dos genes son importantes moduladores de la vía Nodal y son necesarios para la organización asimétrica normal del diencéfalo dorsal y los tejidos derivados del endodermo (Bisgrove et al., 1999; Rodaway et al., 1999; Bisgrove et al., 2000), estas evidencias apoyan las alteraciones observadas en la distribución de órganos que expresan GLUT2.

Estudiar la funcionalidad del GLUT2 de pez cebra usando un modelo in vitro de mamíferos e investigar su capacidad para rescatar las células pancreáticas GLUT2-deficientes

En los mamíferos, la expresión de GLUT2 en el hígado, intestino, riñón y páncreas endocrino está regulada por las variaciones en los niveles de hidratos de carbono producidas por re-alimentación y los estados de ayuno (Thorens, 1996). En particular, en las células β pancreáticas un aumento de los niveles de glucosa circulante resulta en un aumento de la captación mediada por GLUT2 que conduce a la despolarización de la membrana plasmática y la activación de la secreción de gránulos de la insulina (Deeney et al., 2000; Henquin et al., 2003; Thorens., 2011). El uso de modelos transgénicos es de gran utilidad en el estudio del papel fisiológico de GLUT2 y su implicación en la secreción de insulina estimulada por glucosa en los mamíferos. Es por esto que, los ratones transgénicos GLUT2-nulos y SDD eran hiperglucémicos e hipoinsulinémicos, habiendo perdido la capacidad de detección de la glucosa necesaria para la secreción de insulina (Guillam et al., 1997; Thorens et al., 2000; Stolarczyk et al., 2007). Curiosamente, Guillam et al. mostraron que mediante la re-expresión de GLUT2 se recuperaba la secreción de insulina inducida por glucosa (Guillam et al., 2000) demostrando una vez más la importancia de este transportador en el metabolismo de la glucosa.

De modo que nos propusimos establecer un sistema *in vitro* mediante el uso de línea celular de células β de mamífero MIN6 (Miyazaki et al., 1990) con el fin de estudiar las funciones fisiológicas del GLUT2 de pez cebra. Nuestros resultados muestran que hemos sido capaces de bloquear el GLUT2 endógeno conduciendo a una pérdida de la secreción de insulina estimulada por glucosa en las células MIN6. Adicionalmente, se establecieron con éxito las condiciones para la expresión del GLUT2 de pez cebra en MIN6, que fue capaz de aumentar significativamente la absorción basal de glucosa en las células pancreáticas. Además, los resultados preliminares apuntan a un posible aumento en la captación de glucosa en las células que expresan el constructo de GLUT2 de rata.

Por lo tanto, los resultados de esta tesis constituyen el primer paso en el establecimiento de un sistema con un futuro prometedor con el que, próximos experimentos rescatando células con el GLUT2 bloqueado con otros GLUT2 exógenos nos ayudara a entender mejor las propiedades funcionales de este transportador en peces. Además, gracias a las proteínas GFP de los constructos transfectados podremos estudiar las características del tráfico del GLUT2 de pez cebra.

Conclusiones

1. Hemos clonado y caracterizado el primer promotor funcional de GLUT4 en una especie no mamífera. Desde el punto de vista estructural la secuencia de este promotor está altamente conservada entre los peces teleósteos y presenta la mayoría de los sitios de unión conocidos como importantes para la regulación de GLUT4.

2. El promotor de GLUT4 de Fugu está regulado por insulina, PPARγ y estimulación por pulso eléctrico en células de músculo esquelético de mamífero. La estimulación con insulina provocó una reducción significativa de la actividad del promotor de GLUT4 de Fugu. Sin embargo, la actividad del promotor de GLUT4 aumentó en respuesta a la activación por PPARγ y contracción controlada experimentalmente.

3- En embriones de pez cebra a 24 y 48 hpf el ARNm de GLUT2 se detecta en el cerebro y en los conductos pronéfricos. A 72 y 120 hpf se observó un aumento generalizado de la expresión de GLUT2 observándose intestino, hígado, páncreas, túbulos pronéfricos y rombencéfalo.

4- El knockdown del GLUT2 de pez cebra causa anomalías severas en la organogénesis del cerebro, resultando en la formación defectuosa de la unión mesencéfalo–rombencéfalo y del rombencéfalo, así como un retraso generalizado del desarrollo corporal.

5- GLUT2 en embriones de pez cebra juega un papel clave durante el desarrollo de células progenitoras neuronales, especialmente en la generación de neuronas GABAérgicas. Esto sugiere la existencia de una región glucosensora en el rombencéfalo del pez cebra en el cual estas neuronas estarían involucradas en la regulación de la homeostasis de la glucosa..

6- Los embriones deficientes en GLUT2 mostraron afectada la captación de glucosa, un aumento generalizado de muerte celular por apoptosis especialmente localizado en la región cefálica y alteraciones en la distribución de los órganos derivados del endodermo. Así mismo, estas evidencias sugieren que la coordinación defectuosa entre la captación de la glucosa y la demanda de hidratos de carbono durante las etapas tempranas del desarrollo embrionario podrían afectar al desarrollo de los órganos que expresan GLUT2 y a la viabilidad celular.

7- El knockdown del GLUT2 endógeno en células MIN6 lleva a una pérdida de la secreción de insulina glucodependiente, y la sobreexpresión del GLUT2 de pez cebra en células MIN6 resultó en un aumento significativo de la captación de glucosa. Estas evidencias confirman que las células MIN6 son válidas para el estudio del papel funcional del GLUT2 de pez cebra en células β pancreáticas.

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