Understanding the tissue-specific roles of IGF1/IGF1R in regulating metabolism using the Cre/LoxP system

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Abstract

It is clear that IGF1 is important in supporting growth and regulating metabolism. The IGF1 found in the circulation is primarily produced by the liver hepatocytes, but healthy mature hepatocytes do not express appreciable levels of the IGF1 receptor (IGF1R). Therefore, the metabolic actions of IGF1 are thought to be mediated via extra-hepatocyte actions. Given the structural and functional homology between IGF1/IGF1R and insulin receptor (INSR) signaling, and the fact that IGF1, IGF1R and INSR are expressed in most tissues of the body, it is difficult to separate out the tissue-specific contributions of IGF1/IGF1R in maintaining whole body metabolic function. To circumvent this problem, over the last 20 years investigators have taken advantage of the Cre/loxP system to manipulate IGF1/IGF1R in a tissue-dependent, and more recently an age-dependent fashion. These studies have revealed that IGF1/IGF1R can alter extra-hepatocyte function to regulate hormonal inputs to the liver and/or alter tissue-specific carbohydrate and lipid metabolism to alter nutrient flux to liver, where these actions are not mutually exclusive, but serve to integrate the function of all tissues to support the metabolic needs of the organism.

Keywords

IGF1; IGF1R; metabolism; liver; Cre/LoxP

Introduction

Although many tissues produce IGF1, it is now recognized that the hepatocyte is the primary source of circulating IGF1 (LeRoith 2008; Sjogren, et al. 1999; Yakar, et al. 1999). Despite the fact that the hepatocyte is the major contributor to circulating IGF1, mature hepatocytes do not express appreciable levels of the IGF1 receptor (IGF1R). However, IGF1R is expressed during early hepatocyte development (Waraky, et al. 2016) and in hepatocellular carcinomas (Liu, et al. 2011; Scharf and Braulke 2003), a topic beyond the scope of this review. The majority of IGF1 circulates bound to IGF1 binding proteins (IGFBP3 or

IGFBP5) and acid labile subunit (ALS) (Hjortebjerg, et al. 2014). These ternary complexes serve to extend the half-life of IGF1 and regulate its bioavailability (see XXX et al current issue for details). In the healthy liver, growth hormone (GH) signaling through the GH receptor (GHR)/Jak2/Stat5B pathway is required to maintain hepatocyte expression of IGF1, ALS and IGFBP3 (Feigerlova, et al. 2013; Rotwein 2012). Both IGF1 and GH work together to promote longitudinal growth (Wit and Camacho-Hubner 2011), as well as modulate metabolic function in adults, as previously reviewed in detail (Clemmons 2012; Moller and Jorgensen 2009). In brief, IGF1 works in in conjunction with insulin to inform cells of adequate nutrient stores to support protein accretion, cell growth and proliferation. Circulating IGF1 levels are reduced under fasting conditions and type 1 diabetes, associated with elevated GH levels. Given the healthy hepatocyte does not express appreciable levels of the IGF1R, the metabolic actions of IGF1 are thought to be due to extra-hepatocyte actions.

Although a metabolic role for IGF1 is well accepted, there are several challenges in regards to understanding how IGF1 mediates these effects. Although the hepatocyte is the primary contributor to circulating IGF1, it is also locally produced by the majority of cells in the body, where most (except the mature hepatocyte) express the IGF1R. Therefore, the relative role of circulating *vs.* locally produced IGF1 has been difficult to differentiate. In addition, as the name implies, IGF1 shares high homology to insulin, and in fact, in most non-vertebrate species only a single insulin-like molecule is found. It is thought that the insulin and IGF1 genes arose from duplication of the common ancestral gene (McRory and Sherwood 1997). In mammals, it is commonly accepted that the primary action of IGF1 is to promote structural growth, while the primary action of insulin is to regulate nutrient utilization in response to a meal. Despite these primary actions, IGF1 and insulin demonstrate important crossover effects on growth and metabolism (Messina 2010). Some of these effects may be due to the high structural and functional homology of the IGF1R and insulin receptor (INSR) (Siddle 2011). However, this interaction may only be relevant when IGF1 or insulin levels are at patho/supra-physiologic levels or in the context of *in vitro* experiments, where the levels of bioactive IGF1 applied can be many fold that observed *in vivo*, when binding proteins are absent. *In vitro*, it has also been shown that the IGF1R and INSR can interact by forming hybrid receptors or by crosstalk of downstream intracellular signaling pathways, thereby modifying the response to their respective ligands (Belfiore, et al. 2009; Belfiore, et al. 2017). *In vivo*, the relative expression level of IGF1R and INSR varies between tissues and across development stages, and these changes serve to dictate the relative tissue-specific role of IGF1 vs. insulin in regulating metabolic function.

Congenital whole body knockout of IGF1 results in severe growth retardation and, depending on the genetic background, mice die shortly after birth, while others can survive into adulthood (Liu, et al. 1993; Powell-Braxton, et al. 1993). Congenital whole body knockout of IGF1R is embryonic lethal (Powell-Braxton et al. 1993). Therefore, in order to understand the role IGF1/IGF1R play in regulating metabolism, and to separate the action of IGF1/IGF1R from that of insulin/INSR, over the last two decades multiple laboratories have taken advantage of the Cre recombinase/loxP system (Van Duyne 2015) to manipulate IGF1 and IGF1R in a tissue-specific fashion. This review will focus on those studies, as they relate to how IGF1/IGF1R may regulate tissues to alter production of metabolic hormones and/or alter insulin sensitivity to ultimately control substrate utilization. It is important to note that

the phenotype observed in the mouse models described below may not only be due to loss of function of IGF1/IGF1R actions, but also due to the model of the floxed mouse used, including background strain and the position of the loxP sites (Dietrich, et al. 2000; Holzenberger, et al. 2000; Kloting, et al. 2008; Liu, et al. 1998) and the promoter and method used to drive Cre expression (see below and Table 1 for model details). Where the later will dictate time (*i.e.* developmental stage) of gene knockdown and tissue (cell) specificity.

As summarized in the following sections, IGF1/IGF1R can alter extra hepatocyte function to regulate hormonal inputs to the liver and/or alter tissue-specific carbohydrate and lipid metabolism to alter nutrient flux to liver, where these actions are not mutually exclusive, but serve to integrate the function of all tissues to support the metabolic needs of the organism, as illustrated in Fig. 1.

IGF1 can indirectly regulate (hepatocyte) metabolism through modulation of hormonal inputs

Hepatocyte-specific reduction in IGF1 increases GH

The first application of the Cre/loxP system to study IGF1/IGF1R was the work of LeRoith and colleagues who crossbred IGF1$^{fl/fl}$ mice with the hepatocyte-specific albumin promoter-Cre mice (expressed early in embryonic development), referred to as liver IGF1 deficient (LID) mice (LeRoith 2008; Yakar et al. 1999), or the interferon inducible liver/lymphocyte-specific Mx-Cre mice (Sjogren et al. 1999; Wallenius, et al. 2001). In both models, circulating IGF1 levels were less than 20% of controls, and GH levels were elevated, but structural growth was relatively normal. When LID mice were crossbred to ALSKO mice, circulating IGF1 levels were reduced further, leading to reduced bone growth, but not to the extreme observed in mice with whole body knockout of IGF1 (Liu et al. 1993; Powell-Braxton et al. 1993). These studies revealed that locally produced IGF1 is critical to support normal growth during development.

Despite relatively normal growth, adult LID mice exhibit systemic insulin resistance and impaired response of skeletal muscle to acute insulin challenge (Yakar, et al. 2001). Under hyperinsulinemic/euglycemic clamp conditions, LID mice also exhibit impaired insulin-mediated hepatic glucose suppression (Yakar, et al. 2004). Importantly, the alterations in systemic insulin resistance and hepatic glucose output were normalized in LID mice when treated with a GH releasing hormone (GHRH) antagonist, or when crossbred to transgenic mice expressing a GH antagonist (Yakar et al. 2001; Yakar et al. 2004). Like LID mice, mouse models with congenital, liver-specific knockout of the GHR or its downstream effectors, Jak2 or Stat5, have extremely low levels of IGF1 and high levels of GH, associated with systemic insulin resistance (Cui, et al. 2007; Fan, et al. 2009; List, et al. 2014; Mueller, et al. 2011; Sos, et al. 2011). When congenital, hepatocyte-specific GHR knockout mice are crossbred to mice expressing an IGF1 transgene in the liver, GH levels are reduced and systemic insulin resistance normalized (Liu, et al. 2016). Taken together, these studies indicate that liver-derived, circulating IGF1 mediates some of its metabolic effects indirectly by controlling GH secretion, as discussed below in more detail.

Although systemic insulin resistance develops in congenital liver-specific IGF1 knockout mice, as well as mice with liver-specific knockout of GHR signaling, the liver phenotype is dramatically different. LID mice show little change in hepatic lipid content, while mice with defects in hepatocyte GH signaling develop extreme fatty liver. More recently it has been shown that adult-onset loss of hepatocyte GH signaling increases *de novo* lipogenesis and promotes fatty liver (Cordoba-Chacon, et al. 2015), where this action maybe, in part, independent of reduced IGF1 (Liu et al. 2016). Therefore, the lack of hepatic fat accumulation in the LID mouse, compared to models with hepatocyte defects in GH signaling, may be due to high levels of circulating GH acting through intact, hepatocyte GHR to suppress *de novo* lipogenesis.

The metabolic phenotype of mice with hepatocyte IGF1 deficiency is consistent with an increase in GH actions, where GH antagonizes the actions of insulin and enhances lipid oxidation (Moller and Jorgensen 2009). Also, GH can promote white adipose tissue (WAT) lipolysis under physiologic conditions when relatively unopposed by insulin, or at pathophysiologic levels, as in acromegaly (Moller and Jorgensen 2009; Rabinowitz and Zierler 1963; Sakharova, et al. 2008). In the context of normal physiology, these actions of GH are important under fasting conditions, where insulin and IGF1 levels are low and GH levels are elevated, helping to shift nutrient utilization towards lipids, to maintain circulating glucose levels to support neuronal function.

IGF1 works through IGF1R to suppress somatotrope function

The rise in circulating GH levels in the LID mice and in mice with defects in hepatocyte GHR signaling is due to loss of negative feedback inhibition of circulating IGF1 on hypothalamic/pituitary regulation of GH secretion. Specifically, GH is produced by a subpopulation of anterior pituitary cells, called somatotropes. Regulation of somatotrope function is dependent on the hypothalamic neuropeptides, GHRH and somatostatin (SST), which stimulate and inhibit GH secretion, respectively. GH is released into the systemic circulation in a pulsatile fashion, where the pattern of GH release dictates function. GH interacts with multiple target tissues, including the hepatocyte, to stimulate the synthesis of IGF1. Comprehensive reviews of the literature related to GH/IGF1 axis regulation and function, through 1999, are available online (http://www.comprehensivephysiology.com/WileyCDA/Section/id-420628.html). GH output is tightly controlled by negative feedback loops, where GH and IGF1 feedback at the level of the hypothalamus to reciprocally regulate SST and GHRH input to the pituitary somatotrope, to control GH secretion. It remains to be determined what neuronal cell types dictate these changes in GHRH and SST, and if IGF1 working directly through IGF1R is involved. However, IGF1 does act directly at the level of the somatotrope to suppress GH gene transcription in primary pituitary cell cultures in all species tested, including primates (Luque, et al. 2006). It should be noted that pituitary somatotropes express both IGF1R and INSR, but *in vitro* IGF1 exclusively works through the IGF1R, not INSR, to suppress somatotrope function (Gahete, et al. 2013).

The importance of the direct inhibitory action of IGF1 in regulating somatotrope function *in vivo* has been confirmed using the Cre/loxP system. Specifically, IGF1R$^{fl/fl}$ mice were crossbred with mice that express Cre-recombinase only in the GH-producing cells of the

anterior pituitary, rGHp-Cre mice (Luque, et al. 2007), leading to somatotrope-specific knockout of the IGF1R [SIGFRKO; (Gahete et al. 2013; Romero, et al. 2010)]. The gross morphology of the SIGFRKO pituitary was not altered, however pituitary GH and GHRH receptor (GHRHR) mRNA levels were increased and this was associated with an increase in circulating GH and total IGF1 levels. In contrast, somatotrope-specific knockout of the INSR (SIRKO) did not impact GH and GHRHR gene expression but did enhance GH secretory vesicle release, indicating IGF1 and insulin act through unique mechanisms at the level of the pituitary to control GH output. Consistent with the parallel actions of IGF1 and insulin in suppressing GH output, mice with somatotrope-specific knockout of both the IGF1R and INSR have elevated circulating GH levels greater than observed in SIGFRKO and SIRKO mice (Gahete, et al. 2011; Gahete et al. 2013). Of note, although the hypothalamus is commonly thought to be a master regulator of GH output, the fact that circulating GH and IGF1 are elevated in SIGFRKO mice indicates that negative feedback at the level of the hypothalamus cannot fully compensate for the loss of IGF1 negative feedback at the level of the pituitary. Therefore, both central and pituitary regulatory loops are critical in maintaining appropriate GH output.

In the first study that examined the metabolic phenotype of SIGFRKO mice (Romero et al. 2010), when knockouts were compared to pooled controls of different genotypes, body weight was reduced and this was attributed to a decrease in fat mass, without overtly impacting systemic insulin sensitivity. In contrast, in the second report (Gahete et al. 2013), when SIGFRKO mice (IGF1R$^{fl/fl}$/rGHp-Cre) were compared only to their IGF1R$^{fl/fl}$ littermate controls, body weight and body composition did not differ up to 20 weeks of age, when mice were maintained on either a low-fat or high-fat diet. However, in the later study, sex-and diet-dependent changes in metabolic function were observed in SIGFRKO mice that were indicative of mild insulin resistance (Gahete et al. 2013). Taken together, these studies demonstrate IGF1 directly inhibits pituitary production of GH, where IGF1 mediated changes in GH secretion impact systemic insulin sensitivity, that could ultimately remodel body composition. However, it should be noted that the rather mild insulin resistant phenotype observed in SIGFRKO mice, compared to congenital, liver-specific loss of GHR signaling or IGF1 production, is likely due to the anti-insulin effects of elevated GH being off-set by the insulin-sensitizing effects of elevated IGF1.

IGF1 works through the IGF1R to enhance pancreatic β-cell function

Insulin promotes WAT tissue glucose uptake, lipid synthesis and storage (anti-lipolytic) and acts directly on hepatocytes to suppress glucose production, stimulate glycogen and lipid production and suppress VLDL triglyceride release (Titchenell, et al. 2017). Insulin is also required for maximum hepatocyte expression of IGF1, by augmenting IGF1 gene expression and maintaining hepatocyte GHR expression (Dong, et al. 2008; Phillips, et al. 1998). These actions may in part be responsible for reduced hepatic IGF1 production in states of fasting and diabetes type I.

Relevant to this review, intact IGF1R signaling is required to maintain normal β-cell function, based on reports showing rat insulin promoter 2-Cre mice crossbred to two different models of IGF1R$^{fl/fl}$ mice, exhibit impaired glucose tolerance, without impacting

β-cell mass (Kulkarni, et al. 2002; Xuan, et al. 2002). The lack of an impact of IGF1R loss on β-cell mass *in vivo* is somewhat at odds with studies showing IGF1 augments β-cell replication that used cell lines or overexpression models (Chowdhury, et al. 2014; Huang and Chang 2014). These differential effects could be related to the fact that cell lines are not fully representative of β-cells *in vivo* and overexpression of IGF1 may lead to activation of the INSR, which has been shown to be important in establishing β-cell mass (Otani, et al. 2004).

In vitro, islets from β-cell specific IGF1R KO mice had reduced expression of Glut2 and glucokinase, and a reduction in glucose- and arginine-stimulated insulin release. Although the impact of β-cell loss of IGF1R is relatively mild, similar to that reported for β-cell loss of INSR, knockout of both IGF1R and INSR causes early onset overt diabetes (Ueki, et al. 2006). Taken together these observations suggest IGF1 works in conjunction with insulin to establish optimal β-cell function to allow appropriate insulin release in response to nutrient stimulation. Also, early loss of one receptor may in part compensate for the loss of the other. Appropriate models remain to be developed to test how IGF1/IGFIR directly mediates β-cell function in the adult islet.

IGF1 working through the IGF1R supports thyroid hormone production

Thyroid hormone, under fasting conditions, reduces insulin sensitivity, promotes lipolysis and directly enhances hepatic glucose output (Martinez and Ortiz 2017). A direct role in thyroid hormone-mediated hepatic IGF1 production appears to be species specific and concentration dependent. However, IGF1 can directly support thyroid hormone production based on the observation that IGF1R$^{fl/fl}$ mice, crossbred to thyroglobulin promoter/enhancer driven Cre mice showed relatively normal thyroid hormone levels associated with elevated TSH, indicative of subclinical hypothyroidism. Curiously, this was only observed in male mice which showed an increase in weight gain, with age-associated increase in fat mass (Muller, et al. 2011). This direct action of IGF1 on thyroid function may explain in part the subclinical hypothyroidism observed in patients with isolated GH deficiency (Aguiar-Oliveira, et al. 2017). However, it should be noted that the GH-independent actions of IGF1 on thyroid function are somewhat controversial, as recently reviewed (Giavoli, et al. 2017).

IGF1 working through the IGF1R is essential for granulosa cell (estrogen) development and function

Estrogens have profound effects on overall metabolic function, as exemplified by the enhanced prevalence of obesity, insulin resistance, diabetes and fatty liver in postmenopausal compared to normal cycling women (Clegg 2012). The positive metabolic actions of estrogen, at least on controlling excess hepatic fat accumulation, appear to be mediated by extra-hepatic actions, since hepatic lipid accumulation does not differ between liver-specific ERα knockout and control mice, under chow or high-fat fed conditions (Hart-Unger, et al. 2017). Estrogen can enhance hepatic IGF1 production (Venken, et al. 2005), but at high levels estrogens antagonize the actions of GH (Birzniece and Ho 2017).

Recent evidence indicates IGF1 acts directly to modulate granulosa cell development and function. Inhibition of IGF1R signaling *in vivo* and *in vitro* using siRNA or pharmacologic

strategies, blocks the ability of IGF1 to augment FSH-mediated steroidogenic enzyme production in granulosa cells (Zhou, et al. 2013). In addition, granulosa cell knockout of the IGF1R by crossbreeding IGF1R$^{fl/fl}$ mice with mice heterozygous for both the Esr2-Cre and Cyp19-Cre transgenes (Baumgarten, et al. 2017), required for maximal recombination, led to impairment of follicular growth and estrogen production, as well as infertility. Of note, using the same strategy to knockout the INSR in the granulosa cells had no appreciable effect. We might speculate that reduced IGF1 input to granulosa cells during catabolic states may contribute to infertility.

IGF1R and INSR appear to fully compensate for each other to support normal adrenal and Leydig cell development and function (glucocorticoids/testosterone)

Glucocorticoids, derived from the adrenal cortex, stimulate glucose production in the liver, antagonize the actions of insulin and decrease insulin secretion from the pancreatic β-cells (Whirledge and DeFranco 2018). Glucocorticoid receptors (GR) physically interact with Stat5b in the liver to promote IGF1 and ALS (Tronche, et al. 2004), but at high levels can decrease the production of IGF1, in part by acting on the hypothalamus to suppress GH secretion (Ferrau and Korbonits 2015). Androgens have gender dependent effects on metabolism, many of which are mediated by tissue specific conversion to estrogen by aromatase (Bianchi and Locatelli 2018; Birzniece 2018). Androgens work in combination with GH/IGF1 to stimulate skeletal and muscle growth (Giannoulis, et al. 2012).

In order to investigate the direct role IGF1R plays in adrenal cortical and Leydig cell development/function, IGF1R$^{fl/fl}$ mice were crossbred to Cyp11a1 promoter driven Cre mouse (SCC-Cre), which drives Cre expression in the adrenogonadal primordium (Neirijnck, et al. 2018). Knockout of the IGF1R had no appreciable impact on adrenal function and resulted in a modest reduction in testicular size without altering androgen production or fertility. A similar phenotype was observed when the INSR was knockout. However, combined IGF1R/INSR knockout resulted in impaired adrenal cortical and testicular development associated with low glucocorticoid and testosterone production. Due to lack of tissue specificity, it remains to be determined how the impact of receptor loss in each tissue may indirectly impact the other tissue. However, these results clearly indicate that with respect to adrenal cortical and Leydig cell development, the IGF1R and INSR can fully compensate for each other's loss. Nonetheless, appropriate models remain to be developed to directly test how IGF1 directly and independently mediates adrenal cortical and leydig cell function in adults, and how these actions would ultimately impact metabolic function. It should be noted that IGF1 may also support male fertility via regulation of Sertoli cell development (Cannarella, et al. 2018), however Sertoli-specific manipulation of IGF1/IGF1R remains to be explored.

IGF1 works through the IGF1R to support M2-like macrophage activation

It is now becoming appreciated that the macrophage can transition from a pro-inflammatory (M1) to an anti-inflammatory (M2) state, where differentially expressed cytokines impact adipose and liver metabolism. In general the M1 phenotype is associated with insulin resistance (Vergadi, et al. 2017). Of note, M2 macrophages show high expression of IGF1, relative to M0/M1 macrophages (Spadaro, et al. 2017). Knockout of the IGF1R in the

myeloid lineage, which includes macrophages, by crossbreeding IGF1R$^{fl/fl}$ mice with LysM-Cre mice (Spadaro et al. 2017) reduced expression of M2 markers. When these mice were placed on a high-fat diet they became more obese and insulin resistant, compared to their IGF1R intact counterparts. These results indicate IGF1 supports an anti-inflammatory macrophage phenotype that may protect against the development of obesity-associated insulin resistance.

It should be noted here that some reports show an increase in hepatic expression of IGF1R in animal models and patients with chronic liver disease, including non-alcoholic steatohepatitis (NASH). At least in the early stage of the disease, this increase in IGF1R expression is likely due to non-hepatocyte cells types within the liver, which could include resident macrophages (Kupffer cells), infiltrating macrophages, activated hepatic stellate cells, cholangiocytes and vascular endothelial cells. However, very low levels of IGF1R expression in the mature hepatocyte might play a functional role, which could be tested in future investigations using adult-onset, hepatocyte-specific IGF1R knockdown. Nonetheless, treatment with IGF1 has been shown to reduce liver injury and fibrosis, at least in part acting on these non-hepatocyte cells (Takahashi 2017). As liver disease progresses to hepatocellular carcinoma, the hepatocytes dedifferentiate to again express the IGF1R, which is thought to contribute to cancer progression (Liu et al. 2011; Scharf and Braulke 2003) (REF include review in current issue on IGF1 and cancer).

IGF1 can indirectly regulate (hepatocyte) metabolism through altering the development and function of key metabolic tissues

IGF1 works through the IGF1R to directly enhance adipose tissue function

Adipose tissue is essential to store nutrients and control glucose/lipid homeostasis, where lack of adipose tissue (lipodystrophy) is associated with extreme systemic insulin resistance and fatty liver. Diet-induced obesity is also associated with adipose tissue insulin resistance and development of fatty liver (Polyzos, et al. 2017).

The direct impact of IGF1 on adipocyte function, *in vivo*, was first investigated by crossbreeding IGF1R$^{fl/fl}$ mice with aP2 promoter driven Cre mice (Kloting et al. 2008). These mice had increased fat mass and adipocyte size. However, after this publication appeared, it was shown that the aP2 promoter Cre mice were not adipocyte specific, where recombination of the floxed allele could also occur in peripheral and central nervous tissue and the adrenal medulla (Martens, et al. 2010). Based on these observations, subsequent studies using the Cre/loxP system to manipulate floxed alleles in the adipocytes have turned to the adipocyte-specific adiponectin Cre mice. To this end, crossbreeding IGF1R$^{fl/fl}$ mice with adiponectin Cre mice resulted in a reduction in subcutaneous and brown fat mass, reduced expression of lipogenic genes in visceral fat depots, and reduced levels of circulating leptin and adiponectin (Boucher, et al. 2016). However, these changes did not result in appreciable changes in response to glucose and insulin challenge. Of note, knockout of the INSR in adipocytes had a more severe metabolic phenotype, while combined loss of both IGF1R and INSR resulted in lipodystrophy and ectopic accumulation of fat in the liver and muscle (Boucher et al. 2016; Softic, et al. 2016). Although additional follow up studies

are required to understand the full metabolic impact of IGF1 signaling in adipocytes, it is clear the actions of IGF1 are distinct from insulin, where both are required for adipocyte maturation and adult function.

IGF1/IGF1R plays an age-dependent role in muscle development and function

Active muscles preferentially oxidize lipids for energy and promote overall metabolic health (Lundsgaard, et al. 2018). During myocyte development, IGF1R expression is predominant, while in the differentiated muscle INSR expression predominates (O'Neill, et al. 2016). Crossbreeding IGF1R$^{fl/fl}$ mice with mice with mef-2c-73k promoter Cre expressed early in development (Mavalli, et al. 2010) impaired muscle fiber formation and reduced muscle weight, but did not impact glucose homeostasis. However, knockout of muscle IGF1R using the actin promoter (Acta) driven Cre, expressed in differentiated muscle cells (O'Neill, et al. 2015), did not impact normal body weight or muscle mass, but did modestly impair muscle function (wheel running). Muscle-specific INSR had a more pronounced phenotype, while combined IGFIR/INSR deficiency (MIGIRKO) showed severe impairment in muscle mass leading to premature death due to respiratory complications. Surprisingly, MIGIRKO did not impact glucose or insulin tolerance.

In contrast to the muscle-specific knockout models, muscle IGF1 receptor (IGF1R)-lysine-arginine (MKR) mice expressing a dominant-negative mutant IGF1R in skeletal muscle and heart are diabetic with insulin resistance in muscle, liver, and adipose tissue (Fernandez, et al. 2001). Of note, crossbreeding the MKR mice with mice lacking both IGF1R and INSR in the muscle (MIGIRKO) led to exacerbation of metabolic dysfunction which could not be attributed to changes in heart function, suggesting the mutant IGF1R can interact with receptors other than IGFR1, INSR or downstream targets to alter skeletal muscle metabolism (O'Neill et al. 2015).

Similar to skeletal muscle loss of IGF1R, loss of IGF1R in heart using muscle creatine kinase promoter driven Cre mice, did not promote changes in heart function or whole body glucose metabolism. However, by 6 months of age the loss of the INSR led to a deficit in heart function, while combined loss of IGFR and INSR led to alterations in heart glucose metabolism and function, leading to death by one-month of age (Laustsen, et al. 2007). Therefore, insulin signaling, not IGF1 signaling, appears to dominate with respect to controlling aspect of mature skeletal and cardiac muscle function.

Future lessons to be learned from age-dependent, tissue-specific inactivation/activation of IGF1/IGFR signaling

Although tremendous progress has been made in understanding the tissue-specific role IGF1 plays in regulating metabolic function, in the majority of mouse models discussed above, the IGF1 or IGF1R gene is deleted early in development, where compensation by insulin/INSR or other unknown factors may contribute to the metabolic phenotype observed in the adult. Given that metabolic disease typically develops after sexual maturation, due to excess caloric intake and reduced physical activity, it is important to understand how changes in IGF1 production and tissue-specific sensitivity impacts disease progression, in order to

identify key targets for treatment, and prevent off-target effects of pharmacologic reduction or enhancement of IGF1/IGF1R signaling. To this end, animal models are required that allow for tissue-specific, inducible, Cre recombinase-mediated inactivation of floxed alleles at specific ages. As discussed previously, this strategy was first used to examine the impact of hepatocyte loss of IGF1 using the interferon inducible Mx-Cre model (Sjogren et al. 1999; Wallenius et al. 2001). More recently this technology has been applied to generate an adult-onset, whole body knockdown of IGF1R (Francois, et al. 2017). Specifically IGF1R^{fl/fl} mice were crossbred to tamoxifen-inducible ubiquitin promoter driven Cre mice (Ubc-CRE[T2]), and offspring were treated with tamoxifen starting at postnatal day 3.5, 31 or 90 (adults). Complete recombination of the IGF1R^{fl/fl} allele was confirmed in all tissues tested. Specifically focusing on adult-onset loss of IGF1R signaling, circulating GH levels were elevated leading to an increase in IGF1, consistent with the importance of negative feedback of IGF1 to suppress GH production (Gahete et al. 2013; Romero et al. 2010). This was associated with mild hyperglycemia, glucose intolerance and insulin resistance, with reduced fat mass, which may be the consequence of loss of the "insulin-like" effects of IGF1, combined with the antagonist actions of GH on insulin signaling. Interestingly females, but not males, showed reduced fertility, which may be related to loss of IGF1 support on granulosa cell function (Baumgarten et al. 2017; Zhou et al. 2013). Further characterization of this unique model with respect to thyroid, adrenal, immune, adipocyte and muscle, under different physiologic and nutritional conditions, will enhance our understanding as to how IGF1/IGF1R integrates the functions of metabolically relevant tissue to maintain adult metabolic function.

Further application of the inducible Cre recombinase system is required to ultimately understand how raising or lowering IGF1 input regulates the function of adult tissues to achieve metabolic homeostasis. However, a limitation of the inducible Cre recombinase models, as in congenital Cre-mediated knockout models, remains tissue specificity of the promoter. In addition, in some inducible models, low levels of Cre expression may occur, "leak", independent of the inducer. Also, the necessity of repeated treatment of the inducer (tamoxifen, doxycycline, or interferon) can have effects independent of inducing Cre recombinase expression.

Another strategy to induce efficient, age- and tissue-specific knockdown or expression of a transgene is the use of adeno-associated viral vectors (AAV) using tissue-specific promoters, where the serotype of the AAV also preferentially targets some tissues over others. This strategy has advantages over the use of adenoviral vectors because it is more efficient, long lasting and does not evoke an acute inflammatory response. This strategy has been most applied to study the role of factors in hepatocyte metabolism using AAV8-throxine binding globulin promoter (TBGp) driven transgenes. Although this strategy has not yet been directly applied to knockdown IGF1 in adult hepatocytes, it has been used to knockdown the GH receptor. Specifically, adult GHR^{fl/fl} mice were treated with a single injection of AAV8-TBGp-Cre, leading to loss of the GHR exclusively in hepatocytes. As in developmental loss of the hepatocyte GHR (GHR^{fl/fl} mice crossbred to albumin promoter driven Cre), circulating IGF1 levels are dramatically reduced and GH levels are elevated. However, in contrast to the congenital model which is thought to develop fatty liver indirectly due to GH-mediated development of systemic insulin resistance and WAT lipolysis, shifting nutrient

flux to the liver (Cui et al. 2007; Fan et al. 2009; List et al. 2014; Mueller et al. 2011; Sos et al. 2011), mice with adult-onset loss of the hepatocyte GHR developed fatty liver due to enhanced *de novo* lipogenesis, independent of systemic insulin resistance (Cordoba-Chacon et al. 2015; Wolf Greenstein, et al. 2017). Taken together these studies demonstrate the phenotype of tissue-specific, adult-onset loss of gene function, may not replicate that observed in tissue specific, congenital knockout models. Therefore, the AAV strategy can be used in future studies to better understand the hepatocyte-specific role of GH/IGF1 in regulating adult metabolism. Such studies may include, but are not limited to 1) determining if adult-onset loss of hepatocyte IGF1 replicates the phenotype observed in LID mice, 2) differentiating between the direct hepatocyte actions of GH from those mediated by elevation in circulating GH due to loss of IGF1 feedback by knockout of the GHR in the adult hepatocyte, combined with reconstitution of hepatocyte IGF1 expression, 3) directly testing if hepatocyte IGF1R plays any role in regulating adult hepatocyte function.

Although the AAV delivery of transgenes represents a powerful tool, once again the tissue-specificity is dependent on the promoter used to drive transgene expression. Also, AAV does not incorporate into the genome, so if applied to rapidly dividing cells, or the transgene induction enhances cell division, the vector will become diluted in subsequent cell generations. In addition, antibodies do develop to AAVs, not allowing for the sequential application of AAV vectors.

Summary

Although all the Cre/loxP models have limitations, use of these models has allowed us to refine our knowledge regarding how IGF1/IGF1R regulates metabolism, independent of the actions of insulin. It is clear from these studies that IGF1/IGF1R acts on many tissues to fine tune metabolism, where future applications of these strategies will continue to focus our understanding of the age-dependent, tissue-specific role IGF1/IGF1R plays in health and disease.

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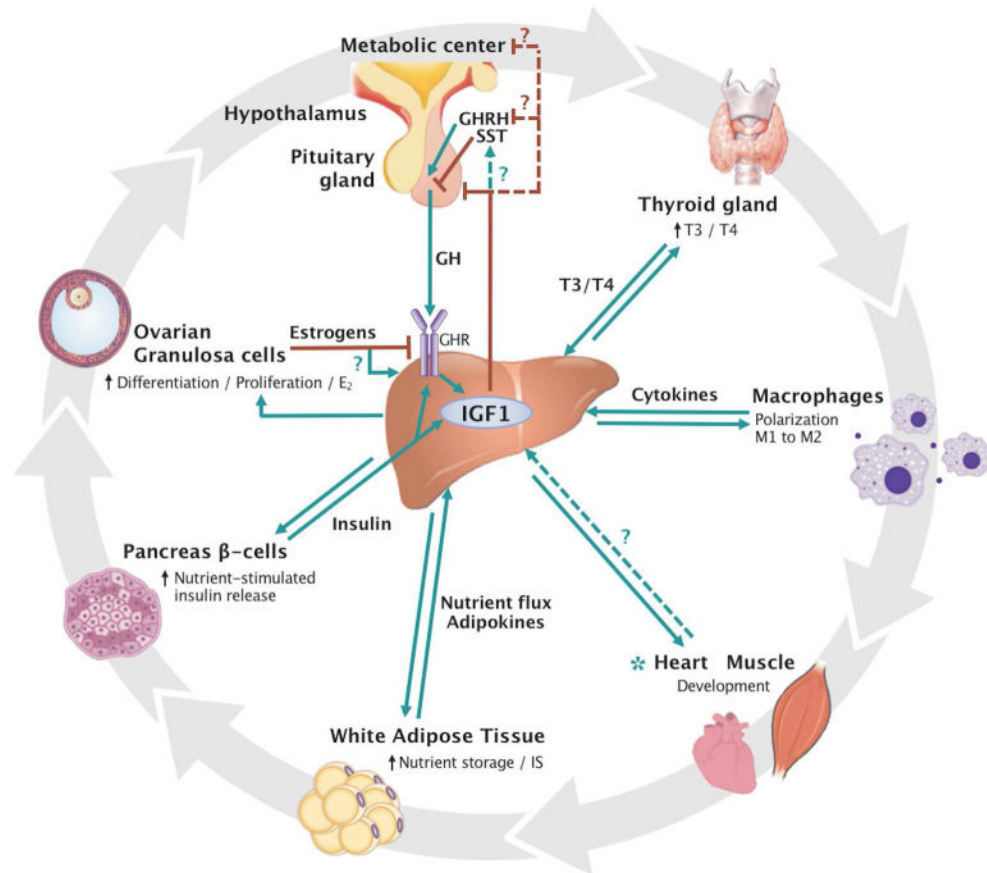
Figure 1.
Summary of the information gained by use of the Cre/loxP system to assess the importance of hepatocyte-specific production of IGF1 and to determine the tissue-specific role of IGF1R on metabolic function. These studies have revealed IGF1/IGF1R can alter extra-hepatocyte function which may regulate hormonal inputs to the liver and/or alter tissue-specific carbohydrate/lipid metabolism to alter nutrient flux to liver, where these actions would not mutually exclusive, but serve to integrate the function of all tissues to support the metabolic needs of the organism (as depicted by the thick gray shaded arrows forming the circle). Solid green lines (with arrowheads) denote a positive action, while red lines with flat heads denote a negative action. Dashed lines indicate pathways not yet explored or questions remain requiring further investigation. * Asterisk denotes direct IGF1 signaling plays a minimal role in how muscle mediates whole body metabolic function.

Table 1

Summary of studies employing the Cre/loxP system to study the role of IGF1/IGF1R signaling

Gene	Tissue	Promotor Driven Cre	Phenotype	Ref
Igf1[a, b]	Whole Body Knockout		-No IGF1/elevated GH -Severe growth retardation -Early death	Liu et al. 1993 Powell Braxton et al. 1993
Igf1[a]	Liver (hepatocyte)	Albumin	-Very low IGF1/elevated GH -Insulin resistance -Minimal growth defect	LeRoith 2008 Yakar et al. 1999 Yakar et al. 2001 Yakar et al. 2004
Igf1[a]	Liver/Lymphocyte	Interferon-inducible Mx1-Cre	-Very low IGF1/elevated GH -Minimal growth defect	Sjogren et al. 1999
Igf1r[c]	Somatotrope	rGHp-Cre	-Elevated GH and IGF1 -Mild insulin resistance	Luque et al. 2007 Gahete et al. 2013 Romero, et al. 2010
Igf1r[d]	Pancreatic β-cell	Insulin promoter 2	-Impaired nutrient mediated insulin release without change in β-cell mass	Kulkarni et al. 2002 Xuan et al. 2002
Igf1r[e]	Ovarian Granulosa Cells	Esr2 + Cyp19	-Decrease granulosa cell differentiation, proliferation and estrogen production	Baumgarten et al. 2017
Igf1r[e]	Leydig Cells and adrenal cells	SCC-Cre	-No IGF1R-specific effect	Neirijnck et al. 2018
Igf1r[c]	Muscle	Actin	-No impact on body weight, muscle mass or metabolism	O'Neill et al. 2015
Igf1r[d]	Heart	Creatine Kinase	-No changes in heart function or whole body glucose metabolism	Laustsen et al. 2007
Igf1r[c]	Adipose Tissue	Adiponectin	-Reduced fat mass -reduced fat synthesis -reduced adipokines -No change on glucose homeostasis	Boucher et al. 2016
Igf1r[c]	Adipose Tissue Brain/ Adrenal Medulla	aP2	-Increased fat mass -Reduced relative brain weight	Klöting et al. 2008
Igf1r[e]	Myeloid Cells (Macrophages)	Lys2	-M2 to M1 polarization -Increased fat mass and insulin resistance in response to high-fat feeding	Spadaro et al. 2017
Igf1r[c]	Thyrocyte	Thyroid-specific thyroglobulin	-Subclinical hypothyroidism -Increased fat mass (males only)	Muller et al. 2011

Superscripts a–e indicate the specific floxed model used:

[a] Liu JL *et al* 1998, *Igf1* exon 4 flanked by loxP sites

[b] Powell-Braxton L *et al* 1993, *Igf1* exon 3 flanked by loxP sites

[c] Kloting N *et al* 2008, *Igf1r* exon 3 flanked by loxP sites

[d] Holzenberger M *et al* 2000, *Igf1r* exon 3 flanked by loxP sites

[e] Dietrich P *et al* 2000, *Igf1r* exon 3 flanked by loxP sites.