Universidade Federal do Rio Grande
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Tese de Doutorado

Importância da ação autócrina/parácrina dos fatores de crescimento tipo-insulina 2 (IGF2) no crescimento muscular e regeneração do zebrafish (*Danio rerio*)

Bruna Félix da Silva Nornberg

Tese defendida no âmbito do Programa de Pós Graduação em Ciências Fisiológicas - Fisiologia Animal Comparada como parte dos requisitos para obtenção do título de DOUTORA.

Orientador: Prof. Dr. Luis Fernando Marins
Co-orientador: Dr. Márcio de Azevedo Figueiredo

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“Vista à luz da evolução, a Biologia é, talvez sob o ponto de vista intelectual, a mais inspiradora e satisfatória das ciências”

Theodosius Dobzhansky

"*Amor de Família é a coisa mais inexplicável do mundo, nem um pai consegue dizer para um filho o quanto o ama, nem o filho sabe dizer ao pai, então eles simplesmente demonstram...*"

Dedico este trabalho aos meus pais, Mara e Renato.

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SUMÁRIO

LISTA DE ABREVIATURAS

GH – Hormônio do Crescimento (somatotrofina)
GHR- Receptor do Hormônio do Crescimento
GHRH- Hormônio Liberador do Hormônio do Crescimento
JAK- Janus Cinase
STAT- Sinais de Transdução e Ativadores da Transcrição
IGF- Fatores de Crescimento tipo Insulina (somatomedinas)
IGF1a- Fator de Crescimento tipo Insulina 1a
IGF1b/IGF3- Fator de Crescimento Tipo Insulina 1b/ 3
IGF2a - Fator de Crescimento tipo Insulina 2a
IGF2b- Fator de Crescimento tipo Insulina 2b
IGFBP- Proteína de Ligação aos Fatores de Crescimento tipo Insulina
IGF1Ra- Receptor de Fatores de Crescimento tipo Insulina 1a
IGF1Rb- Receptor de Fatores de Crescimento tipo Insulina 1b
M6P/IGF2R- Receptor de manose-6-fosfato/ Receptor de Fatores de Crescimento tipo Insulina 2
GrnA- Pró-granulina A
PI3K- Fosfatidilinositol-3-Cinase
Akt- Proteína Cinase B
IRS- Substrato do Receptor de Insulina
mTOR- Alvo mamífero da Rampamicina
p70S6K- Proteína ribossomal 70kDa S6 Cinase
4EBP1- Proteína de ligação ao fator de elongação 4E1
MAPK- Proteína Cinase Ativada por Mitógeno
Shc- Proteína adaptadora-SHC
Ras- GTP Ras ativa
Raf- Proteína Ativada por Mitógeno (MAP) Cinase- Cinase- Cinase
MEK- Proteína Ativada por Mitógeno (MAP) Cinase- Cinase
ERK- Proteína Ativada por Mitógeno (MAP) Cinase
MPC- Células Progenitoras Miogênicas (células miosatélites)
MRFs- Fatores Regulatórios Miogênicos
MyoD/Myf3-Diferenciador miogênico 1
Myf5- Fator Miogênico 5
Miogenina- Fator miogênico 4
Mrf4/Myf6- Fator miogênico 6
ROS-Espécies Reativas de Oxigênio
GFP- Proteína Verde Fluorescente

RESUMO

O eixo somatotrópico, constituído essencialmente pelo hormônio do crescimento (GH) e pelos fatores de crescimento tipo insulina (IGFs), é o principal regulador do crescimento somático em vertebrados. Entretanto, o real papel dos diferentes IGFs na fisiologia de teleósteos ainda não está totalmente esclarecido. Dessa forma, o principal objetivo desta tese foi avaliar a participação dos IGFs sobre o crescimento muscular pós-embrionário e regeneração em zebrafish (*Danio rerio*). Assim, o primeiro estudo desta tese utilizou um modelo de zebrafish GH-transgênico para avaliar a relação entre os IGFs produzidos no músculo e no fígado com o crescimento muscular hipertrófico observado nestes animais. Os resultados demonstraram que a hipertrofia muscular causada pelo excesso de GH pode ocorrer tanto pela produção de IGFs provenientes do fígado quanto pela ação do IGF2b autócrino/parácrino proveniente do músculo. Além disso, é sugerido que a hipertrofia muscular observada nos transgênicos pode ser um resultado do desequilíbrio entre a via proliferativa MEK/ERK e a hipertrófica PI3k/Akt, a favor da última. No segundo estudo, foi avaliada a resposta regenerativa da musculatura esquelética de zebrafish selvagens após exercício de baixa intensidade. Estes experimentos mostraram uma queda no peso e fator de condição dos animais treinados. Porém, após um período de recuperação (72 Horas), os animais conseguiram restaurar o peso e fator de condição, além de aumentar o conteúdo total de proteína quando comparado aos demais grupos. Associado a estes resultados, houve uma alteração no padrão de expressão dos genes dos IGFs e dos fatores regulatórios miogênicos (MRFs) demonstrando que o treinamento e a recuperação têm efeitos distintos na expressão gênica. Ficou evidente também que *igf2a* e *igf2b* são importantes para a recuperação da musculatura de zebrafish após um estado catabólico. Tendo em vista que outros trabalhos já observaram a importância do IGF2b na regeneração de zebrafish, o último estudo desta tese teve como objetivo determinar se a superexpressão do GH pode alterar a capacidade regenerativa da nadadeira caudal através do sistema IGF. Foi observado que o excesso de GH potencializa a regeneração da nadadeira caudal de zebrafish após a segunda amputação através do aumento nos níveis de expressão dos genes *igf2a* e *igf2b*. Em conclusão, fica claro que os IGF2 exercem um papel regulador importante sobre os processos de regeneração e crescimento de zebrafish, o que chama atenção para possíveis aplicações destes fatores em estudos envolvendo as ciências biomédicas e para a aquicultura.

INTRODUÇÃO

Regulação hormonal do crescimento

O crescimento nos vertebrados é influenciado por um conjunto complexo de fatores ambientais, celulares e hormonais que desempenham um papel na determinação de muitas características secundárias, tais como comportamento alimentar, eficiência metabólica, idade reprodutiva e tamanho (Raven *et al.* 2008). A via pela qual o processo do crescimento se desenvolve nestes animais começa com a síntese do hormônio liberador do hormônio do crescimento (GHRH) no hipotálamo, o qual induz a síntese do hormônio do crescimento (GH) na hipófise anterior. O stress, o sono e o exercício podem aumentar a produção de GHRH, que leva ao aumento nos níveis de GH na corrente sanguínea (Kopchick & Andry 2000). O GH liberado na circulação irá atuar em determinados órgãos através da sua associação com receptores específicos (GHR) presentes na membrana das células alvo, ativando genes envolvidos no desenvolvimento das respostas biológicas ao GH (Fig.1). A regulação neuroendócrina negativa do GH está bem definida sendo realizada por um peptídeo secretados no hipotálamo, a somatostatina ou hormônio inibidor do GH (Kopchick & Andry 2000).

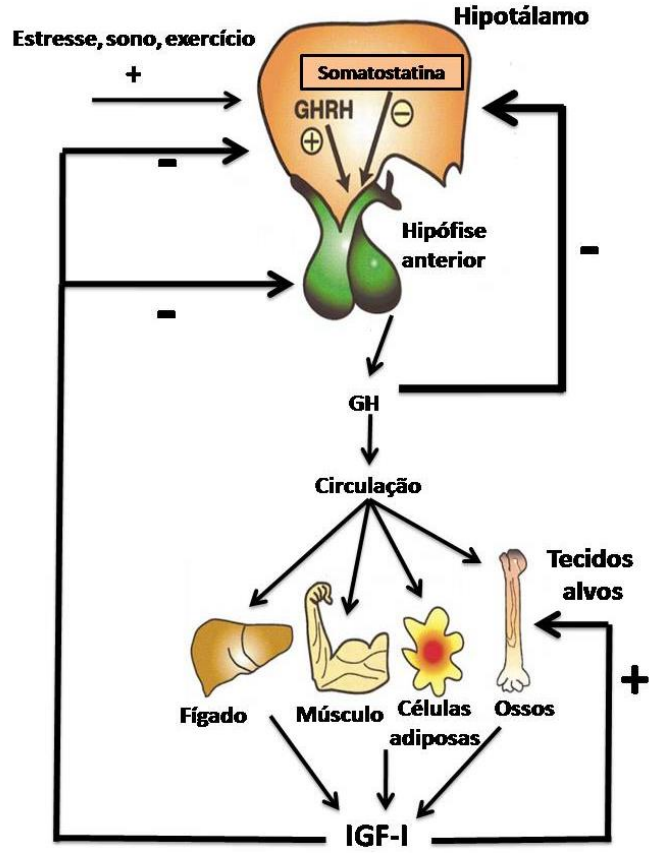


Figura 1: **Secreção e ação do GH em mamíferos**. Fatores como estresse, sono e exercício estimulam o hipotálamo a secretar GHRH, o qual estimula a síntese de GH na hipófise anterior. O GH sintetizado é liberado na circulação onde vai atuar sobre diferentes tecidos alvos estimulando a síntese de IGF-1. O IGF-1 tem efeitos sobre o crescimento através da proliferação e diferenciação celular. Além disso, altos níveis circulantes de IGF-1 e GH inibem GHRH e a liberação do GH. Modificado de Kopchick e Andry (2000).

A ligação do GH com o GHR induz a fosforilação e consequente ativação de membros de uma família de enzimas conhecidas como Janus Kinases (JAKs) comumente associadas à parte intracelular do receptor (Lanning & Carter-Su 2006). Uma vez ativadas, as JAKs fosforilam regiões específicas do receptor ricas em tirosinas, as quais irão servir de sítios de ancoragem para as moléculas conhecidas como Sinais de Tradução e Ativadores da Transcrição (STATs), que também são fosforiladas pelas JAKs, formando dímeros e translocando-se para o núcleo para induzir a expressão de uma ampla faixa de genes, incluindo os fatores de crescimento tipo insulina (IGFs) (Fig. 2) (Wood *et al.* 2005; Duan *et al.* 2010). Estudos envolvendo a incorporação de transgenes para o GH mostraram uma série de efeitos diretos no aumento da expressão de IGF1 e IGF2 sobre vários tecidos e órgãos (Eppler *et al.* 2010). Isto sugere que ambos, IGF1 e IGF2, podem ser os mediadores primários dos efeitos do GH.

Em peixes, a regulação dos diferentes ligantes do sistema IGF pelo GH tem sido discutida. Devlin *et al.* (2014) encontraram um aumento apenas na expressão de *igf1* no fígado de salmão GH-transgênico, sugerindo que o GH possa não exercer um efeito de regulação sobre o IGF2 no fígado desses peixes. Por outro lado, foi observado um aumento de ambos, *igf1* e *igf2*, em diferentes tecidos de carpas comum (*Cyprinus carpio*) injetadas com GH (Vong *et al.* 2003) e tilápias (*Oreochromis niloticus*) GH-trasngênicas (Eppler *et al.* 2010). Estes resultados controversos mostram a necessidade de mais estudos para compreender melhor os efeitos do GH sobre a regulação dos diferentes IGFs em peixes.

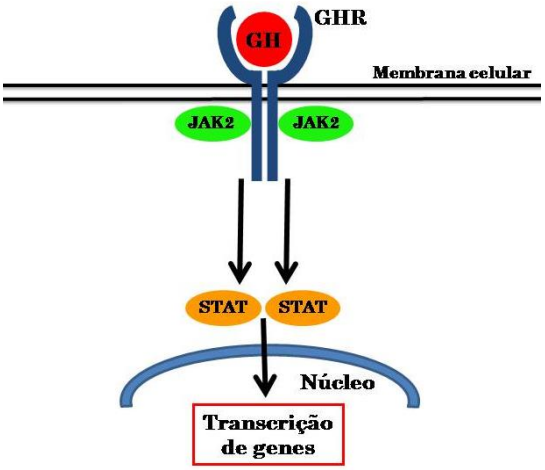


Figura 2: Principal via de sinalização intracelular do hormônio do crescimento (GH) JAK/STAT. A dimerização do receptor do GH (GHR) tipo tirosina cinase ativa as JAK2 por fosforilação. As JAK2 podem ativar outras moléculas sinalizadoras como as STATs, as quais migram para o núcleo e ativam a transcrição de genes como os IGFs. Modificado de Figueiredo (2011).

O sistema dos fatores de crescimento tipo insulina (IGFs) em peixes

O sistema IGF (Fig. 3) desempenha um papel fundamental na regulação do crescimento fetal e pós natal, proliferação e sobrevivência das células, afetando quase todos os órgãos do corpo (Yakar *et al.* 2000; Annunziata *et al.* 2011). Este sistema é composto por três ligantes (IGF1, IGF2 e insulina), seus receptores e as proteínas de ligação ao IGF (IGFBPs) (Annunziata *et al.* 2011). Os IGFs pertencem a uma família de peptídeos que se mantém conservada ao longo da evolução dos vertebrados e acredita-se ser de origem anterior à do GH, aparecendo também em invertebrados (Wood *et al.* 2005).

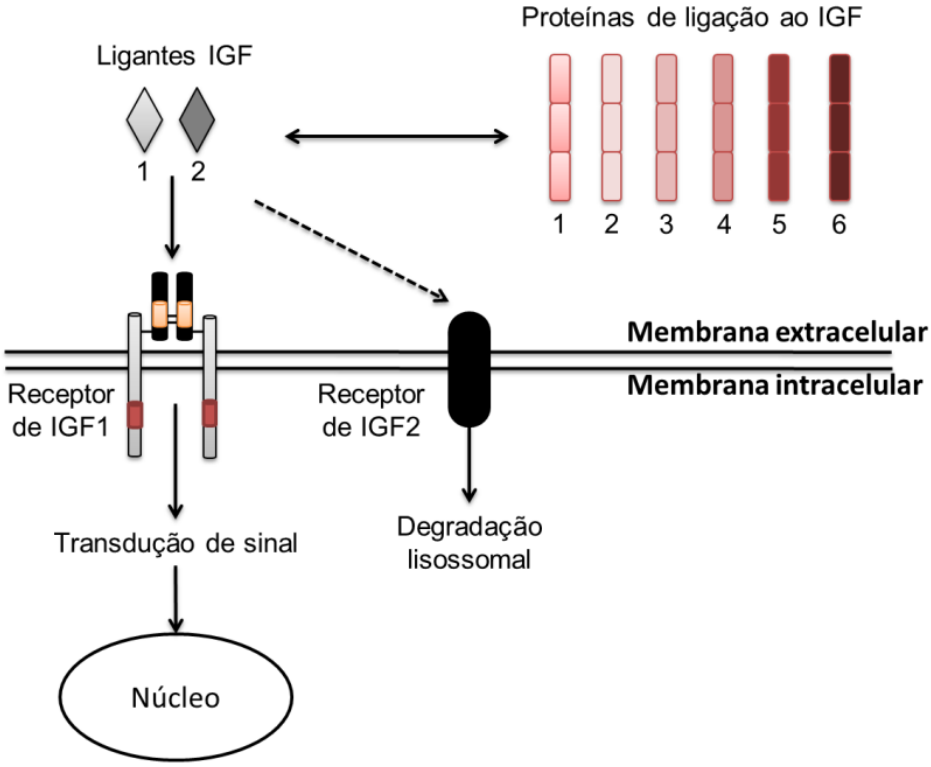


Figura 3: Sistema dos fatores de crescimento tipo insulina (IGFs). Os ligantes IGF1 e IGF2 ativam sua sinalização através de seu receptor do tipo tirosina cinase (IGF1R). O receptor de IGF2 (IGF2R) está relacionado com a degradação dos seus ligantes. As proteínas de ligação ao IGF (IGFBPs) modulam a biodisponibilidade de IGF no ambiente extracelular, desse modo, influenciando as interações dos ligantes com seu receptor. Ilustração adaptada de Wood *et al.* (2005) baseada em um modelo mamífero.

Os ligantes IGF1 e IGF2

A primeira identificação do IGF1 foi como um fator induzido pelo GH que mediava a captação de sulfato na cartilagem, sendo inicialmente referido como um fator de sulfatação (Salmon & Daughaday 1957). Em 1972, Daughaday e colaboradores

propuseram o termo genérico somatomedina a uma substância no soro considerada como um intermediário da ação da somatotrofina (GH) nos tecidos alvos. Durante este período, um novo fator com efeitos semelhantes à insulina foi encontrado no soro humano, cuja atividade não podia ser suprimida pela adição de anticorpos anti-insulina (Froesch *et al.* 1963). Este fator de crescimento tipo insulina, como foi chamado, também mostrou um efeito na promoção do crescimento (Blundell *et al.* 1978). Posteriormente, IGF1 e IGF2 foram purificados, e o IGF1 mostrou ser idêntico a somatomedina (Rinderknecht & Humbel 1978; Klapper *et al.* 1983). Ambas as substâncias foram denominadas como IGF por suas homologias com a insulina e porque seus efeitos no crescimento celular e tecidual predominaram sobre o metabolismo (Daughaday *et al.* 1987).

A existência de um sistema de sinalização do IGF em peixes era incerta até o final de 1980 e início de 1990. Os primeiros esforços para detectar os IGF em peixes foram através de imunoensaios com anticorpos de mamíferos. Resultados contraditórios foram atribuídos à sensibilidade limitada dos anticorpos de mamífero para os IGFs de peixe (para revisão ver Wood *et al.* 2005). Apesar das ambiguidades, fortes evidências da existência de IGFs em peixes foram obtidas através de estudos fisiológicos clássicos. Duan & Inui (1990a, b) relataram um fator de sulfatação GH-dependente no soro de enguia japonesa (*Anguilla japonica*), e demonstraram que IGF-1 humano poderia estimular a captação de sulfato em cartilagem branquial destes animais (Duan & Hirano 1992). Estes estudos sugerem claramente a existência de atividade dos IGFs e receptores de IGF em teleósteos, fornecendo evidências convincentes para a importância da sinalização do IGF ao longo da evolução dos vertebrados. Com o avanço da ciência e a utilização da tecnologia de DNA recombinante, mais interesse e progresso tem sido observado sobre a função da sinalização do IGF em todos os vertebrados, incluindo peixes (Wood *et al.* 2005).

Em peixes, assim como em mamíferos, tanto IGF1 como IGF2 são sintetizados como pré-pró-hormônios. Todos os IGFs contém um sinal peptídico que é removido durante secreção formando um pró-hormônio composto de cinco domínios (B-C-A-D-E). Subsequentemente, ocorre a remoção proteolítica do domínio E originando um IGF maduro (domínios B-C-A-D), contendo entre 68 ou 70 aminoácidos, dependendo da espécie (Wood *et al.* 2005).

Em mamíferos e pássaros, há apenas um gene para cada IGF (*igf1* e *igf2*). O IGF1 é descrito por atuar no crescimento pós-natal (Laron 2001; Kaplan & Cohen

2007), enquanto o IGF2 tem sido, principalmente, relacionado com o desenvolvimento embrionário (Coan *et al.* 2008; Murphy *et al.* 2008). Por outro lado, estudos têm demonstrado que teleósteos possuem mais do que dois genes IGF (Wang *et al.* 2008; Yuan *et al.* 2011). Em 2009, Zou e colaboradores identificaram e caracterizaram quatro distintos genes codificando para IGFs (*igf1a*, *igf1b*, *igf2a* e *igf2b*) em zebrafish (*Danio rerio*) (Fig. 4). Estes genes codificam para quatro peptídeos estruturalmente e funcionalmente distintos. O gene do *igf1a* é ortólogo[1] ao *igf1* de humano, enquanto que, tanto *igf2a* como *igf2b* são ortólogos ao gene *igf2* de humano (Zou *et al.* 2009).

O IGF1 tem sido reportado por exercer diversos efeitos biológicos, principalmente sobre a regulação do crescimento somático, embora existam fortes evidências da ação deste fator de crescimento sobre outros processos fisiológicos em peixes (Wood *et al.* 2005). Por outro lado, o IGF2 parece exercer funções distintas do IGF1. White *et al*. (2009) ao produzir zebrafish *knockdown* para o *igf2a* e *igf2b* indicaram que cada uma destas isoformas desempenha funções diferentes nos estágios iniciais do desenvolvimento embrionário. *Igf2a* foi relacionado com a formação da notocorda durante a segmentação/neurulação, já o *igf2b* é expresso em tecidos adicionais a notocorda, bem como, no cérebro e nos pró-néfrons (White *et al.* 2009). Adicionalmente, Huang *et al.* (2013) observaram um aumento na expressão de *igf2b* em zebrafish adulto durante o processo de regeneração do miocárdio, indicando que, em peixes, o IGF2 pode exercer funções importantes após o desenvolvimento embrionário. Entretanto, há poucas informações sobre as funções biológicas do IGF2 em peixes após o desenvolvimento embrionário.

Outro IGF foi identificado em tilápia (*Oreochromis niloticus*) e zebrafish, sendo denominado como IGF3. Este peptídeo teve suas funções relacionadas com o desenvolvimento das gônadas e reprodução (Wang *et al.* 2008). A origem do gene *igf3* tem sido questionada, podendo este ser originado da duplicação do *igf1* (denominado *igf1b*) e sofrido divergências significantes dos outros genes do *igf* (Zou *et al.* 2009), ou ser um novo gene, como proposto por Wang *et al.* (2008). A manutenção dos quatro genes do IGF no genoma do zebrafish sugere que cada um deles esteja envolvido com funções distintas e indispensáveis.

[1] Genes ortólogos: originados de um único gene do último ancestral comum entre as espécies (frequentemente possuem o mesmo papel biológico dos ancestrais)

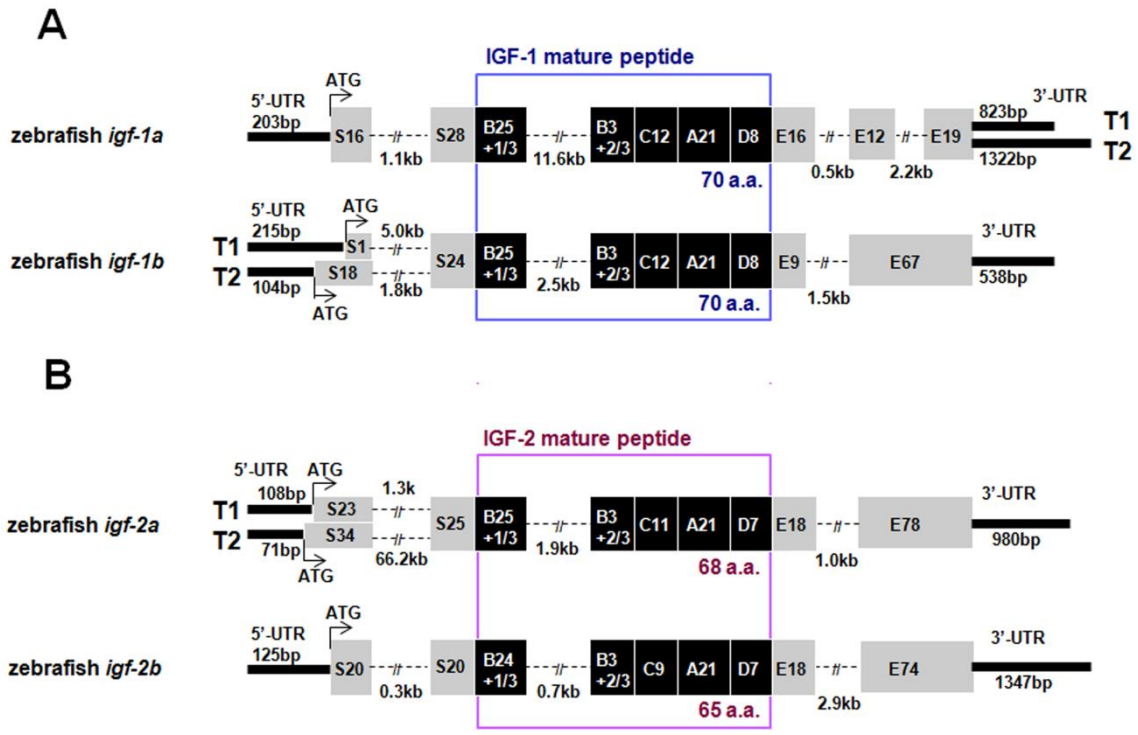


Figura 4: Caracterização molecular dos quatro genes de IGF em zebrafish. (A) a estrutura dos genes IGF1a e 1b de zebrafish. Regiões do peptídeo sinal e o domínio E são apresentadas em caixas cinzentas e os domínios B-C-A-D do peptídeo maduro estão apresentadas em caixas escuras com o número de aminoácidos indicado. As regiões 3' e 5' UTRs estão representadas em linhas cheias com os números de pares de bases (bp) identificados. Sequências de introns estão representadas por linhas tracejadas e estão indicadas em kb. (B) Estrutura dos genes IGF2a e 2b de zebrafish, extraído de Zou *et al.* (2009).

Proteínas de ligação aos IGFs (IGFBPs)

A maioria dos IGFs circulantes é produzida principalmente no fígado e transportada para outros tecidos, atuando como um hormônio. Este peptídeo também pode ser secretado por outros tecidos, incluindo células cartilaginosas e musculatura esquelética, atuando de forma autócrina/parácrina[2] (Laron 2001). Em torno de 99% das moléculas de IGF1 presentes no plasma estão complexadas com uma família de proteínas de ligação ao IGF (IGFBPs), que regulam seu tempo de vida, disponibilidade nos tecidos e a distribuição no corpo (Himpe & Kooijman 2009).

Seis distintas IGFBPs, designadas como IGFBP1 a 6, foram isoladas e caracterizadas em humanos e em outros vertebrados como peixes (Le Roith 2003; Wood *et al.* 2005). Entretanto, um estudo recente usando uma abordagem filogenética encontrou 19 genes parálogos[3] de IGFBP em salmonídeos (Macqueen *et al.* 2013), os quais podem realizar funções que ainda não foram esclarecidas em teleósteos. Dessa

[2] Sinais parácrinos e autócrinos difundem-se localmente para ativar receptores nas células vizinhas (parácrinas) ou em células irmãs e na célula que os liberou (autócrina).
[3] Genes parálogos: originados por duplicação antes ou depois da especiação (podem possuir ou não o mesmo papel biológico)

forma, a presença de IGFBP em teleósteos sugere uma função central desta família de proteínas como reguladoras da sinalização do IGF (Kelley *et al.* 2001).

Shimizu e colaboradores (1999) observaram que a maior parte do IGF1 circulante em salmão prateado (*Oncorhynchus kisutch*) está complexada com uma proteína de 40 kDa, de tamanho semelhante a IGFBP3, á qual a maioria do IGF1 de mamíferos está ligado (Firth & Baxter 2002). Em zebrafish, foi observado elevados níveis de IGFBP3 após a injeção de IGF1 e IGF2, sugerindo que a expressão desta proteína deve ser modulada por estes fatores de crescimento (Chen *et al.* 2004). IGFBP1 foi detectada em vários tecidos durante o desenvolvimento embrionário de zebrafish, mas somente no fígado de animais adultos com níveis baixos de expressão (Maures & Duan 2002). Com relação a IGFBP2, estudos mostraram que a estrutura desta proteína é conservada em dourado (*Sparus aurata*) (Funkenstein *et al.* 2002) e que o perfil de expressão da IGFBP2 durante o desenvolvimento embrionário e nas gônadas indicam que esta proteína pode modular as funções dos IGF sobre desenvolvimento e na reprodução de dourado e carpa comum (Chen *et al.* 2009).

IGFBP4 e IGFBP5 têm sido relacionadas com a regulação positiva dos IGFs no crescimento da musculatura esquelética em peixes. Trabalhos com células miogênicas de salmão atlântico mostraram que IGFs estimulam a expressão de ambas, IGFBP4 e IGFBP5 (Bower & Johnston 2010). Estas proteínas também foram induzidas no músculo durante períodos de realimentação após jejum em diferentes espécies de peixes (para revisão ver Fuentes *et al.* 2013a). Estes resultados sugerem que estas IGFBPs podem atuar como reguladoras positivas da ação do IGF1, sendo consideradas moléculas pró-miogênicas. Além disso, Ren *et al.* (2008) também mostraram que IGFBP5 regula a diferenciação muscular por ligação ao IGF2 e auxilia na auto-regulação do IGF2 em células musculares de mamíferos. Estes resultados mostram que esta proteína também exerce funções importantes sobre o IGF2, porém existem poucos estudos investigando o envolvimento da IGFBP5 e IGF2 no crescimento muscular de peixes. Quanto a IGFBP6, foram detectados altos níveis desta proteína nas células satélites de salmão do atlântico, porém sua expressão diminui durante a miogênese (Bower & Johnston 2010). Também foi observado que a expressão de IGFBP6 diminui durante a realimentação em diferentes espécies de teleósteos, sugerindo que esta IGFBP pode atuar como um modulador negativo das ações do IGF1 em peixes (Fuentes *et al.* 2013a).

O receptor do IGF1(IGF1R) e IGF2 (IGF2R)

O IGF1R pertence à família de receptores do tipo tirosina cinase. Este receptor é um heterotetrâmero composto de duas subunidades α extracelulares e duas subunidades β transmembrânicas (Laron 2001; Himpe & Kooijman 2009). As subunidades α possuem o sítio de ligação ao IGF1 e estão ligadas por pontes de dissulfeto. As subunidades β possuem um pequeno domínio extracelular e um domínio intracelular. A parte intracelular contém um domínio tirosina cinase, que constitui o sinal para o mecanismo de transdução (Annunziata *et al.* 2011) (Fig. 5).

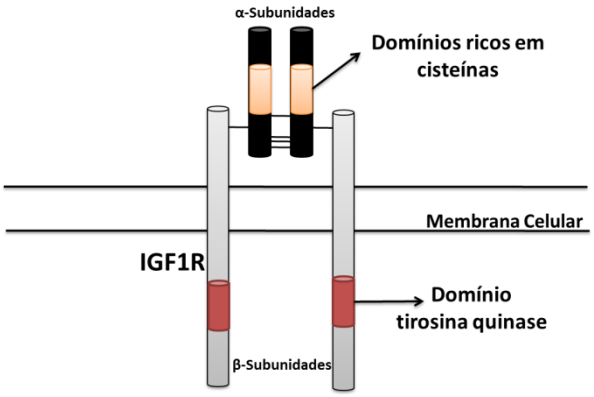


Figura 5: **Estrutura funcional do receptor do IGF1 (IGF1R).** O IGF1R é composto por dois hemireceptores αβ diméricos. As subunidades α são ligadas por pontes de dissulfureto para formar o local de ligação ao IGF na região extracelular. Atividade de tirosina cinase é conferida pelas subunidades β, que atravessam a membrana celular. Modificado de Laron (2001).

A ligação do IGF nas subunidades α leva a ativação das tirosinas cinases intrínsecas nas subunidades β, e subsequente fosforilação cruzada das subunidades β nos resíduos de tirosina, gerando a ativação plena da atividade tirosina cinase deste receptor. A fosforilação dos resíduos de tirosina fora do domínio cinase serve para recrutar outras moléculas sinalizadoras ou inibidoras dos sinais de transdução. Uma das principais vias de sinalização intracelular que emerge após a fosforilação das tirosinas no receptor envolve a ativação da via fosfatidilinositol 3-cinase (PI3K)/proteína cinase B (Akt) como mostra a figura 6 (Himpe & Kooijman 2009).

Resíduos específicos de tirosinas atuam como sítios de ancoragem para proteínas sinalizadoras, tais como a família de IRS (substrato do receptor de insulina). Essas proteínas recrutam a PI3K que leva à fosforilação de fosfolipídios na membrana celular, os quais ativam a Akt (Coolican *et al.* 1997; Annunziata *et al.* 2011). Uma vez ativada, a Akt atua fosforilando o mTOR (alvo mamífero da rapamicina) que ativa a transcrição de vários genes incluindo genes do sistema IGF. A mTOR também fosforila a cinase

p70S6K (proteína ribossomal 70kDa S6 cinase) e a 4EBP1 (proteína de ligação eif4E1) que controlam o aumento na tradução (Glass 2003, 2005). Esta via de sinalização culmina com aumento de síntese protéica, inibição de apoptose, diferenciação, migração e sobrevivência das células (Fig. 6) (Glass 2003, 2005; Clemmons 2009).

Outra cascata de sinalização que pode ser acionada pela ativação IGF1R é a via das proteínas cinases ativadas por mitógenos (MAPK) (Fig. 6), a qual está relacionada ao processo proliferativo e a sobrevivência celular (Coolican *et al.* 1997; Glass 2003; Clemmons 2009). Esta via de sinalização inicia quando as proteínas adaptadoras como Shc se acoplam ao IGF1R após a ligação do IGF no seu sítio ativo, gerando a autofosforilação do IGF1R seguida da fosforilação da Shc, a qual leva a ativação da Ras (GTP ras ativa). A Ras recruta a MAP-cinase-cinase-cinase chamada Raf, a qual ativa a MAP-cinase-cinase (Mek), que, por sua vez, ativa a MAP-cinase (ERK) (Glass 2003, 2005). Esta última fosforila várias proteínas, incluindo outras cinases, bem como, fatores de transcrição. As MAPK são cruciais para a regulação de importantes funções celulares em respostas a estímulos mitóticos (Seger & Krebs, 1995; Robinson & Cobb, 1997).

Estudos sobre regulação das vias de sinalização PI3k/AKT e MEK/ERK na musculatura esquelética sugerem que a via MEK/ERK pode ser inativada durante a hipertrofia (Rommel 1999), como resultado de uma "conversa cruzada" entre as vias PI3k/AKT e MEK/ERK (Zimmermann & Moelling 1999; Moelling *et al.* 2002). Este controle deve ser mantido através da ação de algum participante intermediário das duas vias de sinalização. Em mamíferos, foi sugerido que a proteína Ras possa estar exercendo esta função (Katz & Mccormick 1997).

O receptor de IGF2 ou tipo 2 (IGF2R) é estrutural e funcionalmente distinto do IGF1R. O IGF2R também atua como um receptor de manose-6-fosfato (M6P). O IGF2R/M6P é uma proteína monomérica transmembrana com um domínio extracelular composto por 15 repetições ricas em cisteína. Em mamíferos, IGF2R/M6P tem cerca de 100 vezes maior afinidade para o IGF2 do que ao IGF1 e sua ligação ao IGF2 conduz a degradação lisossomal deste ligante (Kornfeld 1992). As funções biológicas do receptor do IGF2 (M6P/IGF2R) em peixes ainda não estão bem claras. Méndez *et al.* (2001) foram os primeiros a identificar e caracterizar M6P/IGF2R em embriões de truta marrom (*Salmo trutta*) e mostraram que este receptor não possui atividade tirosina cinase. Alguns autores tem sugerido que este receptor possa funcionar como um

"*Limpador*", sequestrando o excesso de IGF e conduzindo para degradação (Kornfeld 1992; Méndez *et al.* 2001; Tsalavouta *et al.* 2009).

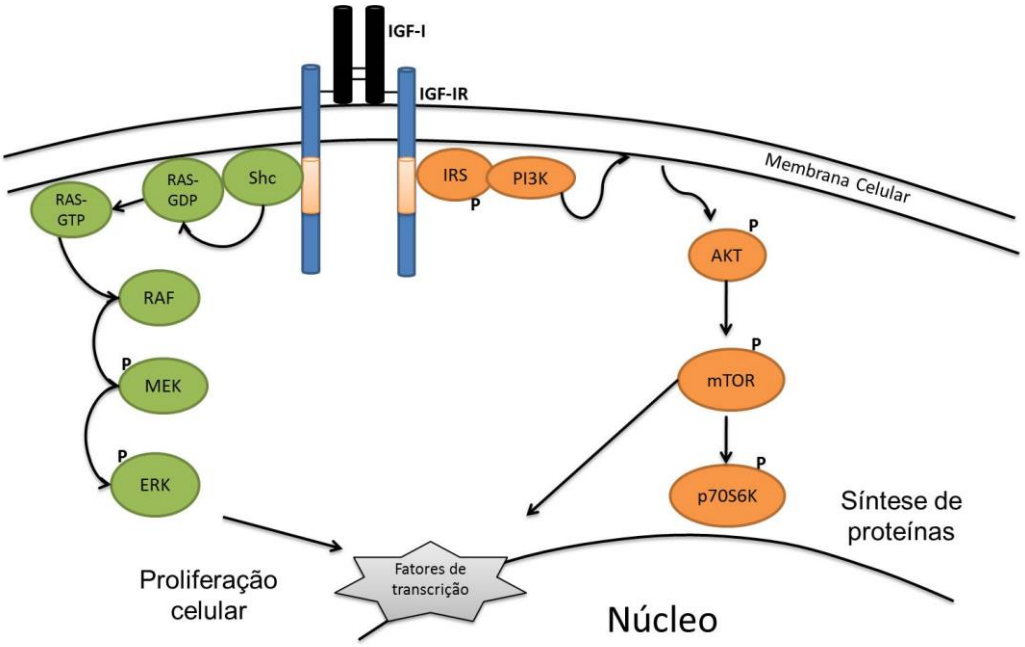


Figura 6: **Principais vias de sinalização intracelular do IGF1R**. Os domínios do tipo tirosina cinase do IGF1R estão indicados na estrutura do receptor em laranja, estes domínios são responsáveis por ativar tanto a via PI3K/Akt como a MEK/ERK. A cascata de sinalização da via PI3K/Akt está representada em laranja e culmina com o aumento da síntese proteica e indução de genes envolvidos com o processo de diferenciação. A via MEK/ERK está representada em verde e tem sido associada com o processo de proliferação celular. Modificado de Himpe e Kooijman (2009).

Com relação às funções do sistema IGF, sabe-se que a ligação de alta afinidade do IGF ao IGF1R desencadeia uma série de respostas fisiológicas como regeneração e hipertrofia da musculatura esquelética (Clemmons 2009), e em condições seletivas, pode promover a diferenciação dos mioblastos, osteoclastos, condrócitos e células neurais (Sara & Hall 1990). Além disso, atua na proliferação de células nas glândulas mamárias durante a puberdade, gravidez e lactação (Hadsell 2004). Há evidencias de que o IGF1 também pode atuar no sistema nervoso exercendo efeitos neuroprotetores em condições patológicas como o Alzheimer (Carro *et al*. 2002) e desempenhando um papel na plasticidade neural e funções cognitivas (Aleman *et al*. 1999; Llorens-Martin *et al*. 2009). Nos peixes, outras funções também têm sido atribuídas a este sistema, incluindo aclimatação osmorregulatória, desenvolvimento reprodutivo e regeneração de diferentes tecidos (Wood *et al.* 2005).

IGFs e a musculatura esquelética

O crescimento muscular esquelético em peixes é mediado pela ação do GH e dos IGFs, que estão envolvidos com a proliferação e diferenciação das células progenitoras miogênicas adultas (MPC), também conhecidas como células satélites. As MPC funcionam como células tronco residentes, sendo mantidas em um estado inativo no músculo maduro. As MPC expressam o fator de transcrição Pax7, o qual é um marcador e regulador do crescimento destas células garantindo sua auto-renovação e manutenção do pool de MPC (Seger *et al.* 2011). Quando a regeneração ou crescimento da musculatura são necessários, estas células rapidamente proliferam-se e diferenciam-se em mioblastos (Fig. 7) (Buckingham 2006). Dessa forma, as MPC são responsáveis pelo crescimento hiperplásico da fibra muscular (crescimento por divisão celular) e crescimento hipetrófico (caracterizado pelo aumento de tamanho das miofibrilas individuais) (Johansen & Overturf 2005).

Muitos estudos sugerem que tanto IGF1 como IGF2 podem promover efeitos durante o crescimento muscular de peixes teleósteos (Castillo *et al.* 2004; Codina *et al.* 2008; Montserrat *et al.* 2012a). Estudos usando diferentes espécies de peixes como truta arco-íris (*Oncorhynchus mykiss*), dourado (*Sparus aurata*) e perca-gigante (*Lates calcarifer*) têm demonstrado que ambos IGFs podem estimular a captação de glicose, a síntese proteica e a proliferação de mioblastos (Degger *et al.* 2000; Castillo *et al.* 2004; Codina *et al.* 2008; Rius-Francino *et al.* 2011; Garikipati & Rodgers 2012a, b; Montserrat *et al.* 2012). Entretanto, ainda não há um consenso sobre a função específica de cada IGF sobre a musculatura esquelética de peixes. Em truta arco-íris, o IGF1 parece exercer um pequeno efeito na indução da diferenciação, já o IGF2 é essencial para este processo em células musculares desta mesma espécie (Gabillard e colaboradores, dados não publicados; revisado por Fuentes et al. (2013a)). Por outro lado, em dourado o IGF2 exerce um efeito mais forte na proliferação de miócitos do que o IGF1 (Rius-Francino *et al.* 2011). Estes resultados chamam atenção para a necessidade de mais estudos sobre as funções dos diferentes IGFs.

Estudos recentes têm mostrado que os IGFs regulam a expressão de genes envolvidos na miogênese, como os fatores regulatórios miogênicos (MRFs): MyoD, Myf5, Miogenina e MRF4 (Garikipati & Rodgers 2012a, b; Jiménez-Amilburu *et al.* 2013). Os MRFs possuem um domínio central conservado conhecido como E-box, o qual é importante para que esses fatores reconheçam uma sequência no DNA presente na região promotora da maioria dos genes músculo-específicos (Edmondson & Olson

1993; Perry & Rudnicki 2000; Johnston 2006). Myod e Myf5 têm sido relatados por estarem envolvidos com os eventos iniciais da miogênese (determinação e proliferação da linhagem miogênica), enquanto Mrf4 e Miogenina seriam responsáveis pelo programa de diferenciação miogênica (Fig. 7) (Rudnicki *et al.* 1993; Rescan 2001; Johnston 2006).

Foi observado em células miogênicas de truta arco-íris que o IGF1 inibe a expressão de *myod1* e *miog* após cinco dias de diferenciação (Garikipati & Rodgers 2012a). Entretanto, após sete dias de diferenciação o IGF1 induz um aumento de Myf5, MyoD e miogenina em células musculares da mesma espécie (Garikipati & Rodgers 2012b). Jiménez-Amilburu e colaboradores (2013) mostraram que o tratamento de células musculares de dourado com IGF1 induz a expressão de miogenina, enquanto o tratamento com IGF2 induz a expressão de MyoD. Além disso, os mesmo autores também observaram que a co-incubação de IGF1 e GH provoca um aumento na expressão de MRF4, enquanto a co-incubação de GH e IGF2 induz a expressão de Myf5 (Jiménez-Amilburu *et al.* 2013). Estes resultados mostram que ainda não está clara a regulação dos diferentes IGFs sobre os MRFs em teleósteos, podendo variar conforme a espécie.

Além dos IGFs, a pró-granulina A (GrnA) também tem sido relacionada com o processo de crescimento e regeneração da musculatura de zebrafish. A GrnA foi descrita inicialmente como um fator de crescimento pluripotente que têm múltiplos papeis biológico, contribuindo na regulação dos estágios iniciais da embriogênese e no reparo de tecidos adultos (Bateman & Bennett 2009; Chitramuthu *et al.* 2010). Recentemente, foi observado que a GrnA regula o crescimento e a regeneração muscular de zebrafish através da manutenção do pool das MPC (Li *et al.* 2013). Estudos recentes mostraram que a GrnA está envolvida com o processo de crescimento muscular através da fusão de fibras, promovendo assim, um crescimento hipertrófico da musculatura esquelética (Hu *et al.* 2012; Li *et al*. 2013)

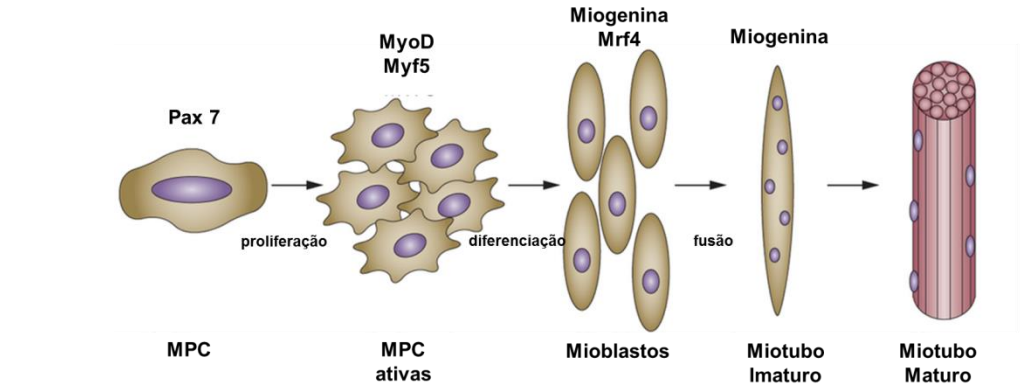


Figura 7: **Principais eventos da miogenese em teleósteos.** Durante o desenvolvimento da fibra muscular e na regeneração muscular, as células Progenitoras Miogênicas (MPC), que se caracterizam pela expressão do fator de transcrição PAX7 requerem a indução de MYOD e Myf5 para a sua ativação e entrada no ciclo celular (proliferação). Subsequentemente são requeridos os fatores Miogenina e MRF4 para a diferenciação destas células em mioblastos e fusão dos mioblastos para formação dos miotubos. Figura adaptada de Perry & Rudnicki (2000) e Salvatore *et al.* (2013).

IGFs e regeneração

A regeneração[4] de órgãos danificados e apêndices é observada em muitos invertebrados e alguns vertebrados como peixes e salamandras, e tem fascinado muitos pesquisadores durante séculos. A capacidade regenerativa difere muito entre os órgãos e organismos, e diversos modelos, que usam diferentes estratégias de regeneração e que oferecem diferentes vantagens técnicas, têm sido utilizados para entender a regeneração (Poss 2010). Dentre esses, o zebrafish tem sido apontado como um excelente modelo, pois este teleósteo tem uma alta capacidade de regeneração associada à disponibilidade de metodologias de manipulação genética bem desenvolvidas (Gemberling *et al.* 2013). Neste contexto, a nadadeira caudal de zebrafish é um dos tecidos mais utilizados em estudos regenerativos, devido sua acessibilidade, arquitetura simples e rápida regeneração (Iovine 2007).

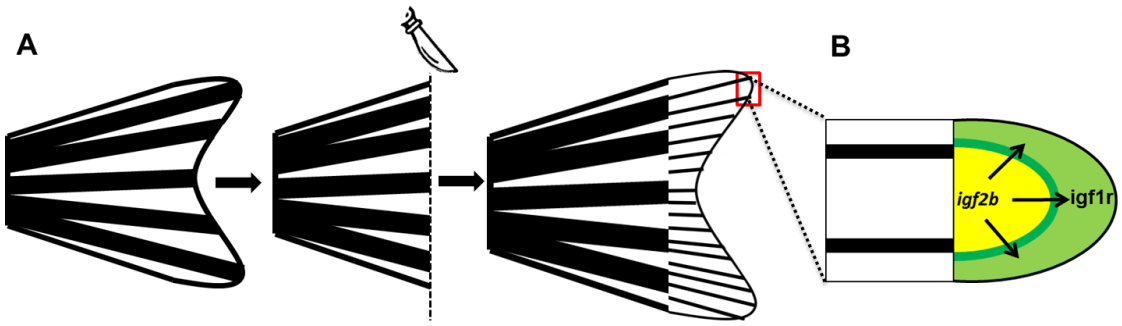
A nadadeira caudal de zebrafish é constituída por 16-18 raios ósseos paralelos intercalados por tecidos moles. Depois de amputação da nadadeira, cada raio, independentemente, forma uma protuberância com capacidade de regenerar por si própria, mesmo após a ablação dos outros raios adjacentes (revisado por Wehner & Weidinger 2015). Assim, cada nadadeira individual fornece múltiplas repetições

[4] Regeneração é comumente definida como a substituição de partes do corpo perdidas por lesão, restaurando sua massa e função. Porém, existe a regeneração homeostática, que se refere à substituição natural de células perdidas por danos menores do dia-a-dia, a morte celular, e ao envelhecimento (Poss 2010).

experimentais. Além disso, a nadadeira pode ser amputada várias vezes, e o processo de regeneração irá se repetir (Azevedo *et al.* 2011).

O processo regenerativo da nadadeira foi descrito por ocorrer em três fases: cicatrização da ferida, formação da blastema[5] e crescimento regenerativo. Durante as primeiras 12 horas após a amputação o ferimento é reparado pela migração rápida de células epidérmicas sobre a superfície da lesão. Os tecidos mesenquimais localizadas na vizinhança da lesão são submetidos à desorganização e aumentam sua proliferação celular. Este processo é seguido pela formação de blastema em até 48 horas após a amputação. A blastema constitui uma zona de crescimento mesenquimal apical que é mantida sob a ferida e a epiderme distal durante a fase de crescimento regenerativo, que é de aproximadamente duas a três semanas (Fig. 8) (para revisão ver Wehner & Weidinger 2015).

Os mecanismos celulares e moleculares que controlam os processos regenerativos são muito complexos e ainda não estão claros. Algumas moléculas sinalizadoras têm sido apontadas por estarem envolvidas com esse fenômeno, incluindo os fatores de crescimento tipo insulina (IGF) (Wehner & Weidinger 2015). A primeira relação do sistema IGF com a regeneração da nadadeira caudal de zebrafish foi encontrada por Jazwinska *et al.* (2007) que identificaram uma indução na expressão do *igf2b* através de uma análise de microarranjo. Posteriormente, este mesmo grupo de pesquisa mostrou que a expressão do ligante IGF2b foi induzida na blastema enquanto que a resposta da sinalização do IGF foi verificada no epitélio apical (Fig. 8) (Chablais & Jazwinska 2010). Assim, ficou evidente que a sinalização do IGF na nadadeira amputada é necessária para que ocorra adequadamente a formação do epitélio da lesão durante o processo regenerativo (Chablais & Jazwinska 2010).



[5] Blastema é uma massa de células mesenquimais proliferativas que se acumulam em certos tecidos após um trauma grave diferenciando-se nas estruturas que foram perdidas (Poss 2010).

Figura 8: Regeneração da nadadeira caudal de zebrafish. (A) Representação esquemática da regeneração da nadadeira caudal de zebrafish após amputação. (B) Sinalização do IGF mediando às interações entre a epiderme da ferida (verde claro) e a blastema (amarelo). A camada basal da epiderme da ferida está representada em verde escuro. As barras pretas representam estruturas esqueléticas. *Igf2b* é expresso no blastema e desencadeia a sinalização através do IGF1R na epiderme da ferida, adaptado de Chablais & Jazwinska (2010).

No entanto, a regeneração não ocorre somente após trauma. A renovação do revestimento intestinal, a geração de novos neurônios no cérebro e a manutenção da pele, cabelo e ossos dependem de regeneração contínua ou cíclica. O sistema IGF também tem sido apontado por estar envolvido com a regeneração de outros tecidos além da nadadeira. Recentemente, Huang *et al.* (2013) mostraram que o ligante IGF2b teve sua expressão aumentada durante a regeneração do coração de zebrafish. Além disso, estes autores também observaram que a inibição da sinalização do IGF1R bloqueia a proliferação dos cardiomiócitos durante o desenvolvimento e regeneração. Estes resultados evidenciam a importância do ligante IGF2b e da sinalização do IGF no desenvolvimento do coração e para a regeneração do miocárdio de zebrafish. Como observado, os estudos envolvendo a regeneração e o sistema IGF são recentes e ainda existem questões a serem esclarecidas sobre a relação desse sistema e a regeneração de diferentes tecidos.

Transgenia do eixo GH/IGF como modelo de estudo do crescimento

Os avanços na biotecnologia nas últimas décadas têm fornecido ferramentas importantes para a manipulação de genes e cromossomos em organismos vivos. Estas novas tecnologias têm sido aplicadas para produção de modelos com grande importância para estudos das vias de sinalização do GH/GHR e suas possíveis interações com outros hormônios e o organismo como um todo (Figueiredo *et al.* 2007b, 2012). A manipulação genética tem sido utilizada com sucesso, a nível experimental, para a produção de peixes transgênicos devido ao fato destes vertebrados superiores apresentarem características reprodutivas e biológicas que permitem uma fácil manipulação dos processos genéticos e fisiológicos nos primeiros estágios da ontogênese (Yan & Sun 2000).

A transgênese envolve a transferência de uma característica de um organismo para outro pela introdução do gene correspondente que codifica tal característica. Desta forma, características novas, estáveis e determinadas geneticamente poderão ser incorporadas ao organismo receptor, com a possibilidade de serem transmitidas para a progênie. A tecnologia de transferência de genes já foi realizada com sucesso em mais

de 35 espécies de peixes (Zbikowska 2003). Grande parte dos peixes transgênicos produzidos teve como alvo o crescimento, por causa de sua aplicação no cultivo e potencial comercialização destes animais. Embora haja controvérsias na aplicação desta tecnologia em escala comercial, recentemente a agência de controle de alimentos e medicamentos (FDA) dos Estados Unidos liberou a comercialização do salmão geneticamente modificado para o GH, produzido pela empresa americana AquaBounty Technologies (Ledford 2015)

Modelo de estudo

O zebrafish (*Danio rerio*; Hamilton,1822) é um peixe da família Cyprinidae, da ordem Cypriniformes. Este teleósteo tem sido amplamente utilizado como um modelo biológico para estudos do desenvolvimento, toxicologia e de transgenia. Dentre as vantagens na utilização desta espécie, destacam-se os baixos custos de manutenção, pequeno espaço necessário, rápido ciclo de gerações, grande número de descendentes, estágios de desenvolvimento bem caracterizados e ovos com córion relativamente fino e translúcido, adequados para os procedimentos de manipulação genética (Udvadia & Linney 2003). Além disso, cabe ressaltar que o genoma completo do zebrafish já foi sequenciado e encontra-se disponível no Banco Mundial de Genes (*GenBank*, http://www.ncbi.nlm.nih.gov), o que facilita sobremaneira os estudos envolvendo esta espécie.

Com o objetivo de estudar os efeitos do eixo GH/IGF no crescimento e seus mecanismos de sinalização intracelular nosso grupo de pesquisa desenvolveu um modelo de zebrafish que superexpressa GH, sendo a primeira linhagem de peixes transgênicos produzida no Brasil (Figueiredo *et al.* 2007a) (Fig. 9). Essa linhagem, denominada F0104, carrega o transgene pcβA-prGH constituído pelo promotor da β-actina da carpa, associado ao gene do GH do peixe-rei marinho (*Odonthestes argentinensis*) (Marins *et al.* 2002). Para a identificação dos indivíduos transgênicos, foi co-injetado o transgene pcβA-GFP, o qual é constituído do promotor da β-actina da carpa, associado ao gene da proteína verde fluorescente (GFP) da medusa *Aequorea victoria*. A co-injeção deste transgene permite a visualização da expressão exógena da GFP, facilitando a identificação *in vivo* dos indivíduos que carregam o transgene para o gene do GH exógeno (Figueiredo *et al.* 2007a). A linhagem F0104, já é reconhecida internacionalmente e integra o "*The Zebrafish Model Organism Database*" (ZFIN - http://zfin.org).

Estudos com diferentes genótipos da linhagem F0104 reportam um aumento significativo na taxa de crescimento de indivíduos hemizigotos e, também, no nível de expressão dos genes do GHR e do IGF1a no fígado (Figueiredo *et al.* 2007b). Embora a manipulação do gene do GH tenha mostrado resultados promissores com relação ao crescimento em peixes, é sabido que o GH atua em vários processos além do crescimento, produzindo efeitos pleiotrópicos sobre a morfologia, fisiologia, metabolismo, imunologia e comportamento (Devlin *et al.* 2006). Ao analisar o sistema de defesa anti-oxidante da linhagem F0104, foi observado um aumento na geração de espécies reativas de oxigênio (ROS), provavelmente relacionado ao efeito anabólico do GH, e como consequência do aumento do consumo de oxigênio e da taxa metabólica (Rosa *et al.* 2008, 2011). Em outros estudos com esta mesma linhagem, Rosa *et al.* (2011) observaram envelhecimento acelerado provavelmente pela diminuição das defesas antioxidantes. Recentemente, Batista *et al.* (2014) demonstraram que o excesso de GH produzido por esta linhagem acarreta em um decréscimo na maturação do sistema imunológico destes animais. Estes estudos apontam para uma série de efeitos fisiológicos colaterais causados pelo excesso de GH o que demonstra a necessidade de compreender melhor a ação desse hormônio e do sistema IGF.

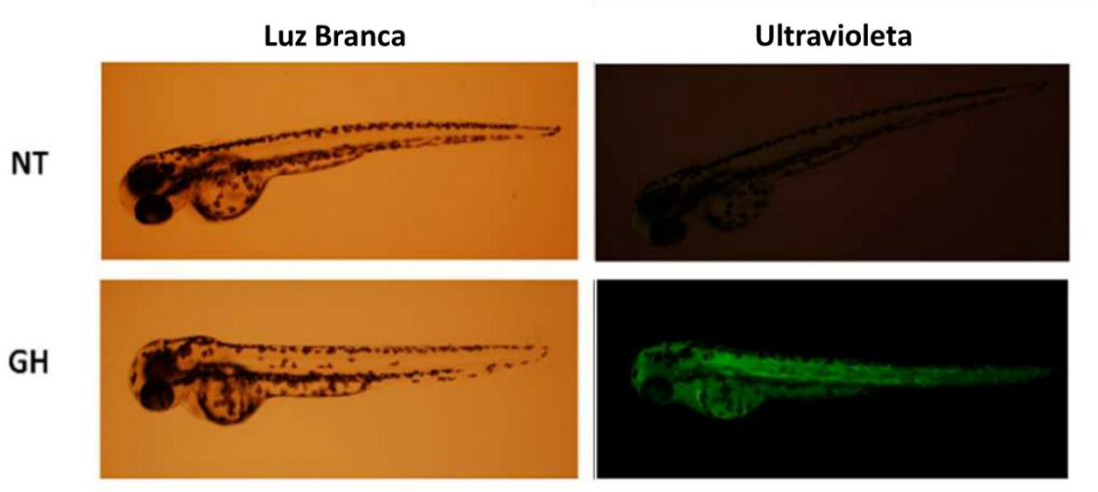


Figura 9: Linhagem F0104. Imagens dos animais não transgênicos (NT) e transgênicos(T) da linhagem F0104 expostos a luz branca e ultravioleta, adaptada de Silva *et al.*, 2015.

OBJETIVO GERAL

Avaliar a participação dos diferentes fatores de crescimento tipo insulina (IGF) sobre o crescimento muscular e regeneração de zebrafish (*Danio rerio*).

Objetivos específicos

- Investigar a relação entre os fatores de crescimento tipo insulina (IGFs) produzidos no músculo e no fígado com a hipertrofia muscular induzida pela superexpressão do hormônio do crescimento (GH) na linhagem F0104;
- Avaliar o envolvimento dos diferentes fatores de crescimento tipo insulina (IGFs) na regeneração da musculatura esquelética de zebrafish após exercício de natação;
- Determinar se a superexpressão do GH pode alterar a capacidade regenerativa da nadadeira caudal de zebrafish através do sistema IGF

MANUSCRITO 1

EXPRESSION PROFILE OF IGF PARALOG GENES IN LIVER AND MUSCLE OF A GH-TRANSGENIC ZEBRAFISH

Autores: Bruna Felix Nornberg, Marcio Azevedo Figueiredo, Luis Fernando Marins

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Expression profile of IGF paralog genes in liver and muscle of a GH-transgenic zebrafish

Bruna Felix Nornberg, Marcio Azevedo Figueiredo, Luis Fernando Marins*

Laboratório de Biologia Molecular, Instituto de Ciências Biológicas, Universidade Federal do Rio Grande – FURG, Rio Grande, RS – Brazil.

*Send correspondence to L.F. Marins. Universidade Federal do Rio Grande - FURG, Instituto de Ciências Biológicas, Av. Itália Km 8, CEP 96203-900, Rio Grande, RS - Brazil. E-mail: dqmluf@furg.br.

Abstract

The objective of this study was to investigate the relationship between IGFs produced in the liver and skeletal muscle with muscle hypertrophy previously observed in a line of GH-transgenic zebrafish. In this sense, we evaluated the expression of genes related to the IGF system in liver and muscle of transgenics, as well as the main intracellular signaling pathways used by GH/IGF axis. Our results showed an increase in expression of *igf1a*, *igf2a,* and *igf2b* genes in the liver. Moreover, there was a decrease in the expression of *igf1ra* and an increase in muscle *igf2r* of transgenics, indicating a negative response of muscle tissue with respect to excess circulating IGFs. Muscle IGFs expression analyses revealed a significant increase only for *igf2b*, accompanied by a parallel induction of *igfbp5a* gene. The presence of IGFBP5a may potentiate the IGF2 action in muscle cells differentiation. Regarding JAK/STAT-related genes, we observed an alteration in the expression profile of both *stat3* and *stat5a* in transgenic fish liver. No changes were observed in the muscle, suggesting that both tissues respond differently to GH-transgenesis. Western blotting analyses indicated an imbalance between the phosphorylation levels of the proliferative (MEK/ERK) and hypertrophic (PI3K/Akt) pathways, in favor of the latter. In summary, the results of this study suggest that the hypertrophy caused by GH-transgenesis in zebrafish may be due to circulating IGFs produced by the liver, with an important participation of muscle IGF2b. This group of IGFs appears to be favoring the hypertrophic intracellular pathway in muscle tissue of transgenic zebrafish.

***Keywords*:** Insulin-like growth factor (IGF) system; growth hormone (GH); transgenic zebrafish; JAK/STAT; MEK/ERK; PI3K/Akt.

1. Introduction

The insulin-like growth factor (IGF) system works in a coordinated form in vertebrates promoting the animal growth, development, metabolism, and longevity, as well as cellular processes leading proliferation, survival, cellular migration and differentiation (Wood et al., 2005). This system includes three ligands (IGF1, IGF2 and Insulin) evolutionarily conserved, their cell surface receptors (IGF1R, M6P/IGF2R and IR) and six high affinity binding proteins (IGFBP-1 to 6) (Le Roith, 2003). IGF1 is the

main mediator in the postnatal actions of the growth hormone (GH) (Kaplan and Cohen, 2007). Most circulating IGF1 is produced in the liver, although it has been demonstrated that this hormone can be synthesized in other organs where it exerts autocrine and paracrine effects (Kaplan and Cohen, 2007; Eppler et al. 2007). Differently, IGF2 shows little dependence on GH and its main function is related to the prenatal development of mammals (Coan et al., 2008; Murphy et al., 2008).

IGF2 has also been detected in several tissues of fish, but its function is still unclear in this group of vertebrates. (Zou et al., 2009) have identified and characterized four IGF genes (*igf1a*, *igf1b*, *igf2a* and *igf2b*) in zebrafish (*Danio rerio*), each encoding a polypeptide with specific characteristics from a structural and functional point of view. These authors also determined that igf1a is orthologous to human *igf1*, whereas *igf2a* and *igf2b* are orthologous to the human *igf2*. It has been recognized that *igf1a* is involved in muscle growth, protein synthesis and myoblast proliferation in fish (Castillo et al., 2004). However, the situation is not so clear about the role of *igf2a* and *igf2b*. The knockdown of these genes in zebrafish demonstrated that the two isoforms have different functions, but both related to the early stages of embryonic development (White et al., 2009). The retention of four IGF genes in zebrafish genome suggests that each one can be involved in different and indispensable functions.

In addition to somatic growth and development, IGFs also play other important functions. Fish IGF1b, initially identified as IGF3, has been related to gonadal and reproductive development (Wang et al., 2008; Zou et al., 2009). Moreover, IGF2b may also be involved in myocardial regeneration process (Huang et al., 2013). In the same sense, IGFs seem to be involved with regeneration and hypertrophy of skeletal muscle (Duan et al., 2010). Several studies have demonstrated that both IGF1 and IGF2 can activate different signaling pathways related to skeletal muscle growth and myoblast proliferation such as JAK/STAT (Himpe and Kooijman, 2009), PI3K/Akt (Glass, 2005, 2003; Rommel et al., 2001), and MEK/ERK (Clemmons, 2009; Codina et al., 2008).

Most studies on the effect and function of the IGF system in fish are performed with cultures of myogenic cells (Codina et al., 2008; Garikipati and Rodgers, 2012; Rius-Francino et al., 2011). In addition, some GH-transgenic fish have been produced and contributed to increased knowledge about the regulation of the somatotrophic axis (Ahmed et al., 2011; Rahman et al., 1998). In order to study the effects of GH/IGF axis on body growth, our research group produced a transgenic zebrafish line (named F0104) overexpressing GH ubiquitously (Figueiredo et al., 2007a). Figueiredo et al. (2007b)

showed increased growth of F0104 transgenic zebrafish, while Kuradomi et al. (2011) demonstrated that GH-transgenesis induced muscle hypertrophy in an independent way of IGF1 locally produced. In this regard, the objective of this study was to investigate the relationship between the liver and muscle IGFs with the GH-induced muscle hypertrophy previously observed in our line of transgenic zebrafish.

2. **Materials and methods**

2.1. GH-transgenic zebrafish

Transgenic (T) and non-transgenic (NT) control fish were obtained from crosses between non-transgenic females and hemizygous GH-transgenic males from F0104 line, following a previously described protocol (Figueiredo et al., 2007a). Briefly, the F0104 line was produced by the co-injection of two transgenes comprised by the carp (*Cyprinus carpio*) β-actin promoter driving the expression of the marine silverside (*Odontesthes argentinensis*) GH coding sequence (Marins et al., 2002) or the green fluorescent protein (GFP) reporter gene. The effect of β-actin promoter can be observed through GFP fluorescence (Figure 1). Taking into consideration that the same promoter controls GH expression, it is assumed that this hormone is being produced with the same location and intensity of GFP. For this study, T and NT zebrafish siblings were reared in a closed circulation water system composed of 15 L aquariums at 28ºC, 14 h light/10 h dark photoperiod, fed with high-protein (47.5%) twice a day, ad libitum. Water quality was monitored once a week, and temperature, pH, nitrogen compounds and photoperiod were maintained according to zebrafish requirements (Westerfield, 1995). When the fish reach seven months of age, twelve males from each group (T=545.5±27.8 mg and NT=353.2±10.3 mg) were euthanized (Tricaine methosulphonate, 0.5 mg mL^{-1}) for molecular analyzes. All experiments were performed as suggested by the Ethics Committee for Animal Use at the Federal University of Rio Grande (FURG, Brazil).

2.2. RNA extraction and cDNA synthesis

Total RNA was isolated from skeletal muscle and liver of six individuals from each group (T and NT) using TRIzol Reagent (Invitrogen, Brazil), following the manufacturer's recommendations. The extracted RNA was treated with DNAse I Amplification Grade (Invitrogen, Brazil). The total amount of RNA was determined with a Qubit Fluorometer and a Quant-iT RNA BR Assay Kit (Invitrogen, Brazil). RNA

integrity was assessed through electrophoresis on 1% agarose gels. For cDNA synthesis, 1 µg of total RNA was reverse-transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Brazil), following manufacturers' protocols.

2.3. Gene expression

Gene expression was analyzed by real-time RT-PCR (7500 Real Time System, Applied Biosystems), using SYBR Green PCR Master MixTM (Invitrogen), according to manufacturer's protocol. Each sample (n = 6) was analyzed in duplicate. Specific primers for each gene (Table 1) were designed using the Primer-BLAST tool from GenBank (http://www.ncbi.nlm.nih.gov). Five-point standard curves of a five-fold dilution series from pooled cDNA were used for PCR efficiency calculation. Gene expression was carried out for 50˚C/2 min, 95˚C/2 min, followed by 40 cycles at 95˚C/15 s and 60˚C/30 s. In this study, four housekeeping genes (elongation factor 1 alpha, *ef1α*; beta-2-microglobulin, *b2m*; beta-actin, *actb1*; and ribosomal protein L13 alpha, *rp113a*) were tested using geNorm VBA applet for Microsoft Excel (Vandesompele et al., 2002). Consequently, we calculated a normalization factor based on the expression levels of the best-performing housekeeping genes. *Ef1α* and *rp113a* were selected as reference genes.

2.4. Western blotting analyses

For Western blotting analyses, protein samples were obtained from skeletal muscle of fish coming from each experimental group. Tissue samples were lysed in a protein homogenization solution (100 mM Tris-HCl, 2 mM EDTA, 5 mM MgCl2 and 250 mL MilliQ water; pH 7.75) and centrifuged for 20 min at 2,000 x g at 4 °C. The supernatant was recovered and centrifuged again for 45 min at 10,000 x g at 4 °C. The supernatant was recovered once more, and its protein content was determined by the Qubit method (Invitrogen, Brazil). Samples were analyzed using SDS-PAGE in 7.5% gels using migration buffer (124 mM Trisma-base, 1 M glycine, 0.5% SDS, and 500 mL MilliQ water; pH 8.3) in miniVE Electrophoresis and Electrotransfer Unit (Amersham Bioscience, Brazil). Each lane contained 30 µg of protein or 5 µL of MagicMark XP Western Standard (Novex, Brazil). Samples were analyzed under reducing conditions (5% 2-mercaptoethanol). After electrophoresis, gels were equilibrated in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol, pH 8.3) for 30 min and electro-transferred (up to 1.0 A, 30 min) in Trans-Blot Turbo Blotting System (BIO-RAD,

Brazil) to a 0.2 μm PVDF membrane (Invitrogen, Brazil), according of the manufacturer's instructions. Membranes were dried, and re-wet with methanol followed by two water washes (20 ml) for 5 min. For the protein immunodetection process, we used the Western Breeze Chromogenic Western Blot Immunodetection System Anti-Rabbit Kit (Novex, Brazil), according to the manufacturer's instructions. The rabbit monoclonal primary antibody used was p44/42 MAPK (Erk1/2) Rabbit mAb (Cell Signaling, Brazil) for total Erk1/2 (used as normalizer), Phospho-p44/42 MAPK (Erk1/2) XP Rabbit mAb (Cell Signaling, Brazil) for phosphorylated Erk1/2, and Phospho-Akt XP Rabbit mAb (Cell Signaling, Brazil) for phosphorylated Akt. The antibodies were used at concentrations recommended by the commercial sources. Films were scanned and specific bands were quantified using 1Dscan EX software version 3.1 (Scanalytics). The band intensity was normalized to the total Erk1/2 band in the same sample.

2.5. Statistical analysis

Data are expressed as means ± SE. Results were compared using the Student's *t* test between-groups. Statistical significance was determined with P values <0.05.

3. Results

To understand the role of liver and muscle IGFs on muscle hypertrophy induced by GH-transgenesis, we analyzed the liver expression of *igf1a, igf1b, igf2a* and *igf2b* genes in our transgenic zebrafish line (F0104). Figure 2 shows that *igf1a*, *igf2a* and *igf2b* genes were up-regulated in transgenics by 11.6, 7.9 and 4.4 times, respectively. The expression of *igf1b* was not different between T and NT. Furthermore, we analyzed the expression of IGFs, IGF receptors and IGF binding protein 5a (*igfbp5a*) in muscle tissue. These results are shown in Figure 3 and it was observed a 35% decrease in expression of *igfr1a* with a concomitant increase of 1.25 times for *igfr2* in transgenic fish. Regarding muscle IGFs expression, only *igf2b* was increased about three times in transgenics.

Taking into consideration that the induction of IGFs expression is dependent on activation of intracellular signaling pathways by which GH exerts its effects, we analyzed the expression of two genes related to JAK-STAT pathway: *stat3* and *stat5a* The results shown in Figure 4 demonstrate that these two genes have distinct liver

expression profiles between T and NT. Liver *stat3* expression was down-regulated in approximately 60%, while *stat5a* was up-regulated by 2.7 times in T zebrafish. No differences were observed between T and NT in muscle tissue for both genes.

PI3K/Akt and MEK/ERK signaling pathways were assessed for level of phosphorylation of its main intermediaries. The results of the Western blotting analyses for muscle tissue (Figure 5) indicated a decrease of 65% in phosphorylation of MEK/ERK pathway in transgenics. No differences were observed in the phosphorylation pattern of the PI3K/Akt pathway between T and NT.

4. Discussion

The regulatory mechanism of muscle growth in fish via GH/IGF axis has been well studied and several studies have suggested that both IGF1 and IGF2 promote growth effects in muscle of teleost fish (Castillo et al., 2004; Codina et al., 2008; Montserrat et al., 2012; Vong et al., 2003). However, a number of questions about the functionality of IGF paralog genes and the importance of endocrine or autocrine/paracrine IGFs produced in liver and muscle still need to be answered (Fuentes et al., 2013). Such questions cannot be answered by studies using cell cultures and in vivo models become indispensable. In this sense, our line of transgenic zebrafish seems an interesting in vivo model to evaluate the effect of endocrine IGFs produced in the liver versus IGFs locally produced in muscle tissue. This transgenic line overexpresses an exogenous GH gene and has accelerated growth with concomitant muscle hypertrophy (Figueiredo et al., 2007b; Kuradomi et al., 2011).

To evaluate the effects of GH overexpression on hepatic production of IGFs, we measured the transcriptional levels of *igf1a*, *igf1b*, *igf2a* and *igf2b* in the liver of transgenic fish. The results of gene expression analysis showed that three (*igf1a*, *igf2a* and *igf2b*) out of four analyzed IGFs in the liver were significantly increased in the transgenic group. Only *igf1b* did not vary between T and NT. Increased transcription of hepatic *igf1a* in a GH-dependent manner is not new. Similar results have been observed in other GH-transgenic fish such as coho salmon (Devlin et al., 2014) and tilapia (Eppler et al., 2007, 2010), reinforcing the action of hepatic IGF1a as major mediator of the biological effects of GH. Indeed, among all genes analyzed in this study, hepatic *igf1a* showed the highest transcriptional response (11.6 fold induction) to GH-transgenesis. In the same direction but not in the same magnitude, hepatic *igf2a* and

igf2b showed significant responses (7.9 and 4.4 fold induction, respectively) to the GH overexpression. Other studies using GH injections in fish have observed the same response from the liver with respect to the production of IGF2, as reported to Japanese eel (Moriyama et al., 2008), grass carp (Yuan et al., 2011) and common carp (Vong et al., 2003). All together these findings point to the liver as the main producer of different IGFs in response to excess circulating GH. Considering that the liver releases all these hormones into the bloodstream, the question arises: what is the effect of each circulating IGF on skeletal muscle tissue?

One way to assess the role of a protein hormone released into the bloodstream on a particular target tissue is to quantify the expression of its membrane receptor. Thus, we compared the gene expression of *igf1ra*, *igf1rb* and *igf2r* in muscle of the T and NT groups. The first result that calls attention in the muscle of transgenic fish is a decrease of 35% in IGF1a receptor expression (*igf1ra*). Whereas the liver is probably releasing huge amounts of IGF1a into the bloodstream, this result points to a decrease in sensitivity of transgenic muscle to the circulating IGFs. On the opposite way is muscular expression of *igf2r*, which was slightly increased in transgenics. Although this seems contradictory, Méndez et al. (2001) suggest that this receptor works as a "clearance" receptor, since IGFs and insulin are not capable of stimulating its tyrosine kinase activity. In this regard, the decrease of *igfr1a* along with the increase of *igf2r* indicates a negative response of the muscle tissue in relation to the excess of circulating IGFs. Furthermore, analysis of muscle IGFs showed a different expression profile from that observed in the liver. Only *igf2b* had a significant 3-fold increase in transgenics. Additionally, *igfbp5a* has been considered a molecule that promotes the differentiation of muscle cells by binding to and switching on the IGF2 auto-regulation loop (Ren et al., 2008). In the same way as for *igf2b*, *igfbp5* had a significant increase in muscle of transgenics. In fact, increased expression of muscle *igf2* in a GH/IGF-dependent manner has already been reported in fish. Studies with cyprinids such as common carp (Vong et al., 2003) and grass carp (Yuan et al., 2011) have shown that muscle growth is under the influence of both endocrine IGFs as much of the autocrine/paracrine IGF2. Additionally, Jiao et al. (2013) report that autocrine IGF2 plays a more important role than IGF1 in mouse myoblast cells. Taking into account the results of gene expression, it is clear that the liver and muscle respond differently to GH-transgenesis and IGF2b may be directly or indirectly related to GH-induced muscle hypertrophy. A further

question is how is the intracellular signaling in these two tissues differentially impacted by excess GH?

IGFs have their transcription regulated by GH, mainly through the activation of the JAK/STAT signaling pathway (Lanning and Carter-Su, 2006). Studies in mammals identified STAT5b binding sites in the IGF1 promoter and have shown that GH can regulate the transcription of igf1 by STAT5b (Chia et al., 2006; Wang and Jiang, 2005; Woelfle et al., 2003). In order to understand the regulation of IGFs expression through the JAK/STAT pathway, we analyzed the expression of *stat3* and *stat5a* in the liver and muscle of transgenics. While transgenics did not alter the expression of both *stat3* and *stat5a* in muscle, liver reacted with significant alterations in both genes. This suggests tissue-specific responses in regard to intracellular signaling via the JAK/STAT pathway due to excess circulating GH in transgenic fish. Thus, these results may explain the altered expression of *igf1a*, *igf2a* and *igf2b* in liver, but does not explain the observed changes in muscle for *igf2b*. Although we have not analyzed the phosphorylation levels of JAK/STAT pathway intermediates, it is likely that muscle of transgenic fish is using alternative routes of intracellular signaling to control the expression of genes related to GH/IGF axis.

Both IGF1 and IGF2 have been well characterized by activating the MEK/ERK and PI3K/AKT pathways in skeletal muscle of various species of fish (Castillo et al., 2006; Codina et al., 2008; Fuentes et al., 2011; Montserrat et al., 2012). Besides, GH can also activate MEK/ERK and PI3K/AKT as alternative pathways (Lanning and Carter-Su, 2006). This way, we evaluated both pathways through phosphorylation level of its main intermediates, comparing the experimental groups T and NT. The phospho-Akt concentrations were not changed in the muscle of transgenic fish. However, we detected a significant decrease in the amount of phospho-ERK, which may be related to the actions of circulating GH or IGFs on muscle tissue. It is known that the PI3K/Akt signaling pathway is more related to cell differentiation and protein synthesis processes, which form the basis of muscle hypertrophy (Clemmons, 2009; Glass, 2003). On the other hand, the MEK/ERK pathway is associated with the proliferation of myoblasts (Jones et al., 2001) and muscle differentiation (Fernández et al., 2002; Li and Johnson, 2006). In this context, we consider a hypothetical scenario where muscle hypertrophy observed in GH-transgenic zebrafish can be a result of an imbalance between the proliferative pathway (MEK/ERK) and hypertrophic (PI3K/Akt), in favor of the latter. It is likely that this antagonistic control is maintained through the action of some

intermediate participating of the two signaling pathways. This function has already been suggested to Ras protein in mammals (Katz and Mccormick, 1997).

In conclusion, this study provides new information on the regulation of muscle growth in zebrafish. It has been shown in zebrafish that GH controls the growth of muscle tissue through both the production of endocrine IGFs (from the liver) and by autocrine/paracrine action of muscle IGF2b. These results call attention to a greater significance in IGF2b function in muscle growth when stimulated by GH. However, for fish as well as other vertebrates, the question of the importance of circulating and local IGFs in promoting muscle growth has not been fully resolved. Thus, the development of transgenic zebrafish lines with constitutive overexpression of different IGFs in muscle and liver could be valuable tools to contribute to the knowledge of the functions of each of these growth factors on muscle growth.

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Table

Table 1. Analyzed genes and primers used for real-time qPCR.

Genes	Primers sequence	Efficiency	Amplicon	GenBank
Igf1a	F: 5'-CAGGCAAATCTCCACGATCTC-3'	100.0	60 bp	NM131825
	R: 5'-TTTGGTGTCCTGGGAATATCTGT-3'			
Igf1b	F: 5'-GCAGCTCGTAGCGGTGGTCC-3'	96.6	66 bp	NM001115050
	R: 5'-TCCACGCACACAACACTGGTCT-3'			
Igf2a	F: 5'-TGCCAAGCCGGTGAAGTCGG-3'	100.0	51 bp	NM131433
	R: 5'-ACCTGCAGCGAGGTGGAGGAA-3'			
Igf2b	F: 5'-ACCTGCCAAGTCAGAGAGGGACG-3'	100.0	63 bp	NM001001815
	R: 5'-GCGGGCATCACTGGAATGACCTT-3'			
Igf1ra	F: 5'-GATCCAAAGAGCAGGGCTCC-3'	100.0	88 bp	NM152968
	R: 5'-GCCATCCCATCCGCTATCTC-3'			
Igf1rb	F: 5'-GGTCTAGCAAACAGAGGCGA-3'	101.8	132 bp	NM152969
	R: 5'-CCAGCCGCTTGAAATTACCG-3'			
Igf2r	F: 5'-GACTGATGGCAGGAAGTGGT-3'	103.4	84 bp	NM001039627
	R: 5'-CCTTGCACACCGTCAGTACA-3'			
Igfbp5a	F: 5'-GCACCCACCCATTGATCGT-3'	85.0	241 bp	NM001126463
	R: 5'-CCTTCTGCACGGACCAAATTC-3'			
Stat3	F: 5'-TGGGAACGGCTTCCCTGGGA-3'	99.9	77 bp	NM131479
	R: 5'-GAGAGTCGAGCGTGCGAGGC-3'			
Stat5a	F: 5'-AAATTGGCGGCATCACTATAGC-3'	90.7	59 bp	NM194387
	R: 5'-CCTTTCCCCTGCTTTGTTAGG-3'			
Ef1α	F: 5'-GGGCAAGGGCTCCTTCAA-3'	100.0	54 bp	NM131263
	R: 5'-CGCTCGGCCTTCAGTTTG-3'			
Rp113a	F: 5'-TCTGGAGGACTGTAAGAGGTATGC-3'	102.9	148 bp	NM212784
	R: 5'-AGACGCACAATCTTGAGAGCAG-3'			
B2m	F: 5'-GCCTTCACCCCAGAGAAAGG-3'	100.5	101 bp	NM131163
	R: 5'-GCGGTTGGGATTTACATGTTG-3'			
Actb1	F: 5'-GCTGTTTTCCCCTCCATTGTT-3'	100.5	60 bp	NM131031
	R: 5'-TCCCATGCCAACCATCACT-3'			

Figure legends

Figure 1. Transgenic zebrafish (*Danio rerio*) expressing both GH (growth hormone) or GFP (green fluorescent protein) under control of carp (*Cyprinus carpio*) β-actin promoter.

Figure 2. Relative gene expression in liver of non-transgenic (NT) and GH-transgenic (T) zebrafish (*Danio rerio*). Data are expressed as the mean ± SE (n = 6). Statistical differences (*t* test, $P < 0.05$) are denoted by asterisks.

Figure 3. Relative gene expression in skeletal muscle of non-transgenic (NT) and GH-transgenic (T) zebrafish (*Danio rerio*). Data are expressed as the mean ± SE (n = 6). Statistical differences (*t* test, $P < 0.05$) are denoted by asterisks.

Figure 4. Relative gene expression of Signal Transducer and Activator of Transcription-3 (*stat3*) and Signal Transducer and Activator of Transcription-5.1 (*stat5a*) in liver (a) and skeletal muscle (b) of non-transgenic (NT) and GH-transgenic (T) zebrafish (*Danio rerio*). Statistical differences (*t* test, $P < 0.05$) are denoted by asterisks.

Figure 5. Western blotting of Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) and Phospho-Akt (Ser473) in skeletal muscle of non-transgenic (NT) and GH-transgenic (T) zebrafish (*Danio rerio*). Total p44/42 MAPK (Erk1/2) was used as the internal control. Asterisk represents statistically significant difference (*t* test, $P < 0.05$; n = 4).

Figures

Figure 1

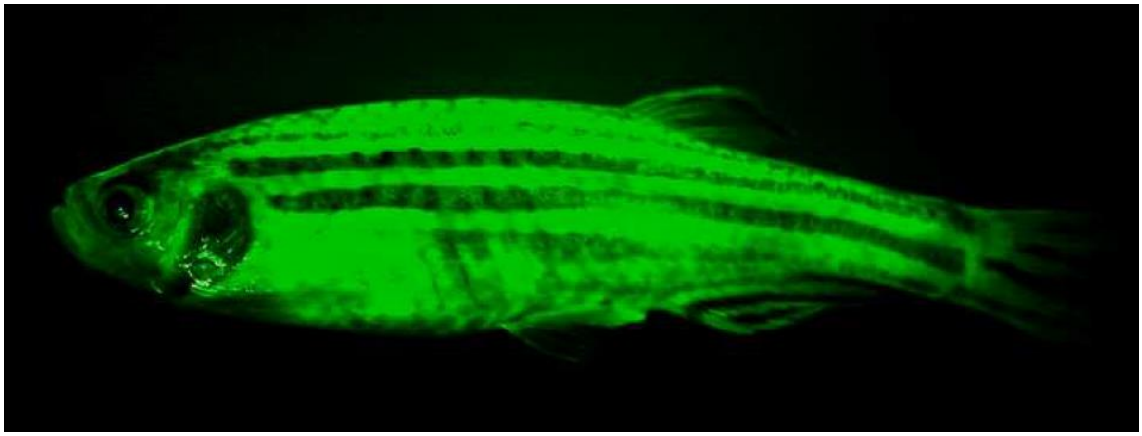


Figure 2

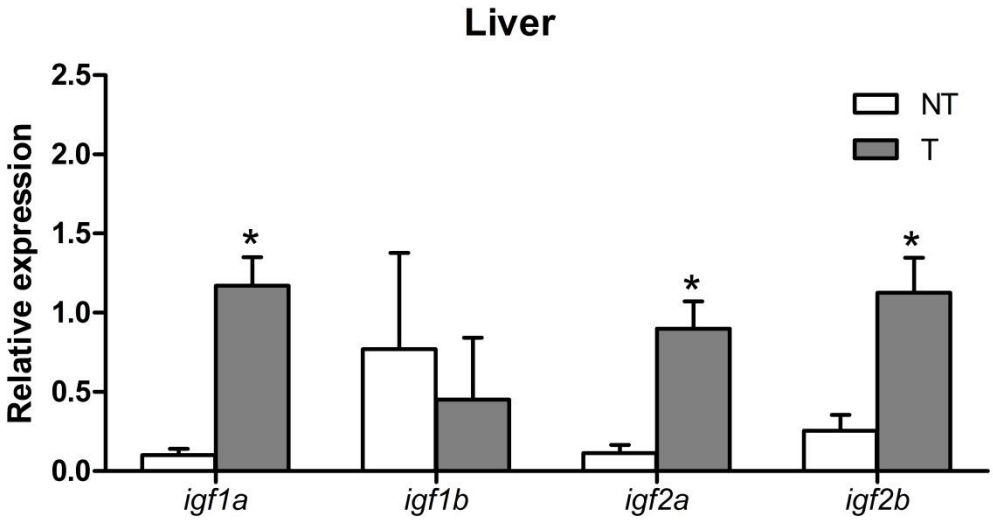


Figure 3

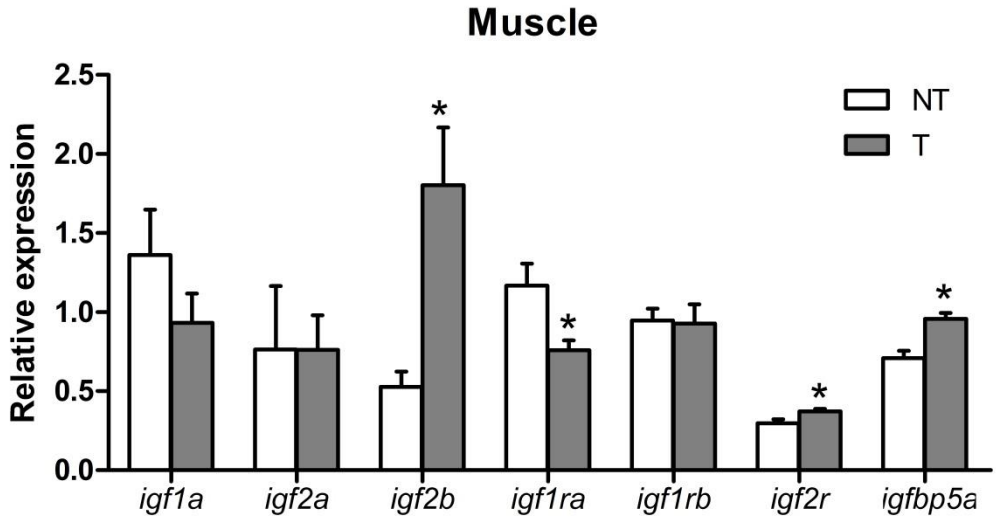


Figure 4

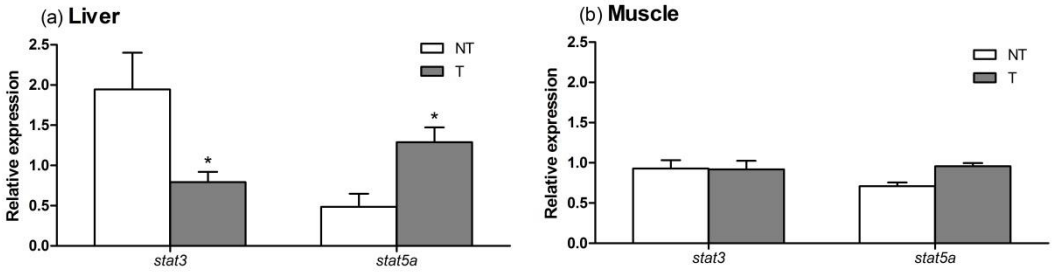
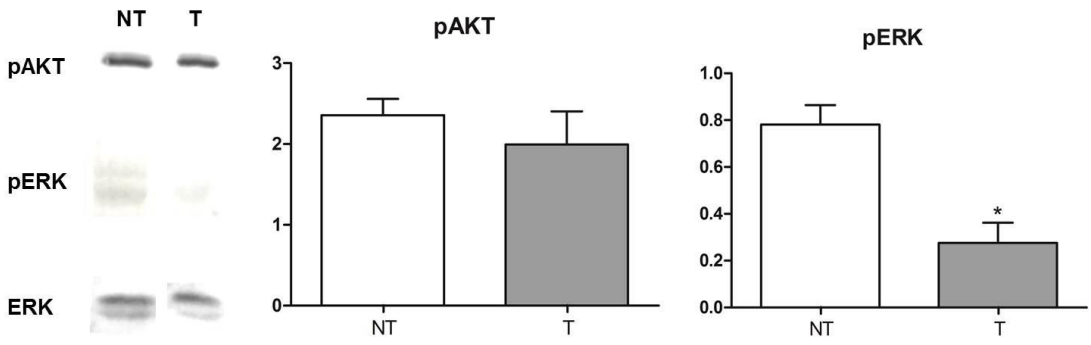


Figure 5



MANUSCRITO 2

ROLE OF INSULIN-LIKE GROWTH FACTOR 2 (IGF2) IN SKELETAL MUSCLE REGENERATION AFTER SWIMMING EXERCISE

Autores: Bruna Felix Nornberg, Mateus Tavares Kutter, Ana Cecilia Gomes Silva, Antonio Sergio Varela Junior, Carla Amorim Neves Gonçalves, Marcio Azevedo Figueiredo and Luis Fernando Marins

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Title: **Role of insulin-like growth factor 2 (IGF2) in skeletal muscle regeneration after swimming exercise**

Authors

Bruna Felix Nornberg[1], Mateus Tavares Kutter[1], Ana Cecilia Gomes Silva[1], Antonio Sergio Varela Junior[2], Carla Amorim Neves Gonçalves[3], Marcio Azevedo Figueiredo[1] and Luis Fernando Marins[1*]

[1]Laboratório de Biologia Molecular, Instituto de Ciências Biológicas, Universidade Federal do Rio Grande – FURG, Rio Grande, RS – Brazil.
[2]Laboratório de Morfologia, Instituto de Ciências Biológicas, Universidade Federal do Rio Grande – FURG, Rio Grande, RS – Brazil.
[3]Laboratório de Fluxometria, Instituto de Ciências Biológicas, Universidade Federal do Rio Grande – FURG, Rio Grande, RS – Brazil.

[*]Send correspondence to L.F. Marins. Universidade Federal do Rio Grande - FURG, Instituto de Ciências Biológicas, Av. Itália Km 8, CEP 96203-900, Rio Grande, RS - Brazil. E-mail: dqmluf@furg.br

Abstract

Skeletal muscle has a significant capacity to regenerate in response to changes in contractile activity. The insulin-like growth factors (IGFs) plays an important role in the muscle growth and regeneration of vertebrates. Current knowlege of IGFs modulation in muscle regeneration in fish after exercise are not clear. Therefore, this work aimed to evaluate IGFs involvement in zebrafish skeletal muscle regeneration after swimming exercise. Were evaluated body weight, condiction factor, fiber quantity and diameter and gene expression in untrained, trained and recovered (72 hours) fish. Weight and condition factor in trained fish decreased, suggesting a catabolic state for this group. On the other hand, recovered fish restored weight, condition factor and muscle total protein content. In addition, recovering also increased total fiber number and proportion of thick fibers. Regarding gene expression, the hierarchical cluster analysis showed that *igf1a*, *igf2a igf2b* genes have distinct expression profiles. There was no change in *igf1a* gene expression, while *igf2a* and *igf2b* were induced in the recovered group when compared

to trained fish. However, it should be noted that *igf2b* was also induced when compared to the untrained group, suggesting greater *igf2b* involvement in muscle regeneration than other IGF system ligands. The *grna* gene had the same expression profile of *igf2b*, highlighting that both are important in muscle growth and regeneration. Besides, an increase in *myod* and *myf5* gene transcripts in the recovered group when compared to trained fish. Such result seems to be related to the increased cell proliferation observed in these fish. In conclusion, our results indicate that i*gf2a* and *igf2b* are important in the zebrafish skeletal muscles recovery after a catabolic state generated by moderate swimming exercise.

Keywords: swimming exercise; skeletal muscle; insulin growth factors (IGFs); myogenic regulatory factors (MRFs)

Introduction

In teleosts, as well as other vertebrates, exercise can lead changes in skeletal muscle growth rate and morphology (Davison and Goldspink 1978; Dougan 1993; Palstra et al. 2010). For this, skeletal muscle developed a significant capacity to regenerate in response to changes in contractile activity. This muscle's regenerative capacity comes from specialized cells, myogenic progenitor cells (MPCs), also called satellite cells (Bryson-Richardson and Currie 2008). The MPCs acts as resident stem cells and are kept in an inactive state in the mature muscle (Buckingham 2006). This process, known as myogenesis, is highly complex and regulated by growth factors and transcription factors, which control the expression of several genes involved in this process.

Myogenesis is highly conserved among vertebrates, and requires the synchronized participation of four myogenic regulatory factors (MRFs): MyoD, Myf5, myogenin and MRF4 (Johnston 2006). These MRFs are transcription factors with a basic helix-alpha-helix domain (bHLH), which is responsible for recognizing the target genes promoter involved in MPC growth and differentiation (Edmondson and Olson 1993; Perry and Rudnicki 2000; Johnston 2006). Myod and Myf5 are involved in the early events of myogenesis (determination and proliferation of myogenic lineage), while MRF4 and myogenin are responsible for myogenic differentiation program (Rudnicki *et al.* 1993; Rescan 2001; Johnston 2006). Beside the MRFs, the MPCs also express the

Pax7 transcription factor, which is a marker and a growth regulator of these cells, ensuring its self-renewal and maintenance of MPCs pool (Seger *et al.* 2011).

The growth, as well as the skeletal muscle regeneration, are also regulated by the growth hormone axis (GH)/ insulin-like growth factors (IGF) (Musarò et al. 2001a; Castillo et al. 2004; Wood et al. 2005; Kaplan and Cohen 2007; Clemmons 2009). This axis is composed by GH, produced in the anterior pituitary and released into the circulation, which can induce IGF1 and IGF2 synthesis in target tissues (Ohlsson et al. 2000; Kaplan and Cohen 2007; Annunziata et al. 2011; Fuentes et al. 2013b). In mammals, IGF1 is described as operating in postnatal growth (Laron 2001; Cohen and Kaplan 2007), while IGF2 has been related mainly with the embryonic development (Coan et al. 2008; Murphy et al. 2008). On the other hand, in teleost both IGF1 and IGF2 have been reported for showing effects on muscle growth process (Castillo et al. 2004; Codina et al. 2008; Montserrat et al. 2012a). Moreover, in zebrafish (*Danio rerio*) were identified four different IGF genes which encodes for different peptides (IGF1a, IGF1b, IGF2a e IGF2b) (Zou et al. 2009). However, the importance of these paralog genes in muscle growth and regeneration has not been fully clarified yet. In addition to IGFs, progranulin A (GrnA) has also been linked to growth and regeneration processes in zebrafish (Li et al. 2013). GrnA was initially described as a pluripotent growth factor that has multiple biological roles, contributing to the regulation of early stages of embryogenesis and adult tissue repair (Bateman and Bennett 2009; Chitramuthu et al. 2010). Recently, it was found that GrnA regulates zebrafish growth and muscle regeneration by maintaining the MPCS pool (Li et al. 2013).

Zebrafish has been widely accepted as a multidisciplinary model of vertebrates (Patton et al. 2001; Gemberling et al. 2013), being appointed recently as an excellent model for studying the physiology of exercise (Palstra *et al.* 2010, 2014). In order to increase the knowledge on exercise-induced muscle regeneration in fish, it was investigated the relationship of different IGFs with the zebrafish skeletal muscle regenerative process after swimming exercise.

Material and Methods

Ethics

Experiments complied with the current law of the National Council for Animal Experimentation (CONCEA – Brazil) and were approved by the Ethics Committee for

Animal Use at the Federal University of Rio Grande (CEUA – FURG, proc. no. 23116.005403/2015-38).

Fish

A total of 34 male zebrafish, six months old, were obtained from a pet store and were of genetically mixed origin. Fish were anesthetized in a solution of 0.1 mg ml-1 tricaine, measured (28.5 ± 0.6 mm) and weighted (241.15 ± 13.65 mg). Later, the fish were randomly distributed into two groups and kept in a recirculating aquaculture system, at average temperature of 28 °C (27.3 ± 0.4 °C), with photoperiod of 14 light hours and 10 dark hours, these conditions were assessed daily. The feeding was carried out supplying commercial diet, twice a day (5% biomass). The water quality was monitored once a week, pH, nitrogen compounds. All parameters were maintained according to requirements for zebrafish (Westerfield 1995).

Exercise training

To conduct the swimming trained, fish (n = 17) were transferred onto a swimming tunnel with continuous flow and water recirculation (Fig. 1), which was built based on Plaut (2000). The water inside the tunnel was kept under the same conditions of the cropping system. The trained fish were exercised for 1 hour, once a day, and six days a week. The initial speed was about 5 cm s^{-1} (equivalent to 1.75 BL s^{-1}, where BL is the body length), the speed was increased by 5 cm s^{-1} every two days until reaching a speed of 15 cm s^{-1} (5.2 BL s^{-1}) at the end of the first week. The training lasted 21 days, and during the second week the fish were trained to 20 cm s^{-1} (7.0 BL s^{-1}) and in the last week at 30 cm s^{-1} (10.5 BL s^{-1}). The maximum speed of this program corresponds to approximately 50% of the critical speed that the zebrafish supports (56 ± 4.8 cm s^{-1}; Plaut, 2000). Therefore, this training was considered low to moderate intensity. After the end of the training protocol 10 fish (trained group) were euthanized (tricaine, 0.5 mg ml^{-1}). The remaining fish (n = 7) have gone through a 72 hours recovery period without training (recovered group). The control group fish (untrained, n = 17) went through the same procedure in the training tunnel, but with the equipment off. At the end of the experiment all fish were euthanized (tricaine, 0.5 mg ml^{-1}) and muscle was dissected for further analysis.

Growth analyses

At the end of the training protocol, fish were euthanized (tricaine, 0.5 mg ml^{-1}) and weighted. Prior to sampling, the fish were fasted for 24 h. The average weight was compared among the three experimental groups (untrained, trained and recovered). In addition, the total length of each fish was measured and the factor condition (K) was calculated using the formula $K = (WL^{-3}) \times 10^3$, where W is the weight in milligrams and L is the total length, in millimeters.

Lactate content and total protein quantification

Muscle samples from five fish of each group were weighed and homogenized in a 1 mg:9 µL mixture of tissue: phosphate buffer (KH_2PO_4 100 mM; K_2HPO_4 100 mM; EDTA 1 mM; PMSF 10 µM; pH 7,2). The samples were homogenized, centrifuged for 20 min at 8,000 x g and kept at 4 °C. The supernatant was removed and a 1 uL aliquot was used to measure the total protein content using Qubit Fluorimeter and Quant-iT Protein Assay Kit (Invitrogen, Brazil), following the manufacturer's recommendations. The resulting values were normalized by sample weight and used in the following analyzes in milligrams of protein per milligram of muscle tissue (mg^{-1} mg). The lactate content was measured using a commercial reagent kit (Kovalent) by spectrophotometry using a microplate reader (EL-x808IU, BioTek Instruments). Results were normalized by the total protein content.

Histological analyses

Skeletal muscle tissue from five fish in each treatment was immediately fixed with 2% paraformaldehyde and stored in 70%ethanol. Samples were dehydrated through a series of ethanol concentrations (80, 90, 95, 95, 100 and 100%), diafanized in xylol (twice) and embedded in paraplast xtra (LEICA ASP 200S instrument, Germany).Transversal histological sections of muscle fibers (4µm) were obtained through an automatic microtome (Leica RM 2255). Sections were stained with hematoxylin-eosin (HE) and histological images were captured using a digital camera DP72 (Olympus, Japan) coupled to an optical microscope BX53 (Olympus, Japan).The total number of muscle fibers persection was analyzed and the smallest diameter of each muscle fiber was determined using Image J software (US National Institute of Health, available at http://rsb.info.nih.gov/ij/). Fibers were classified according to its diameter as follows: ≤18 µm (fine fibers) and>18 µm (thick fibers).

Gene expression

Total RNA was extracted from skeletal muscle of five individuals from each group using Trizol reagent (Invitrogen, Brazil). The quality and amount of RNA were analyzed with a Qubit Fluorometer using a Quant-iT RNA BR Assay Kit (Invitrogen, Brazil). The resulting RNA samples were analyzed by agarose gel electrophoresis. The extracted RNA was then purified with DNAse I Amplification Grade (Invitrogen, Brazil) to avoid contamination with genomic DNA. For cDNA synthesis, 1 µg of total RNA was reverse-transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Brazil). The obtained cDNA was used as a template for gene amplification using primers (Table 1) designed using the Primer-BLAST tool from GenBank (http://www.ncbi.nlm.nih.gov). Quantitative Real Time PCR (qPCR) was performed with an ABI 7500 System (Applied Biosystems, Brazil) using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Brazil). qPCR reactions with cDNA serial dilutions were conducted for all primers to test the efficiency of the reactions. The PCR program consisted of 40 cycles of 95 ºC for 15 s and 60 ºC for 30 s after an initial cycle of 50 ºC for 2 min and 95 ºC for 2 min. The synthesized cDNA (1 µL) was used for the qPCR reactions in a total volume of 10 µL. Each sample reaction was performed in triplicate. Expression of target genes were normalized by expression of elongation factor 1 alpha (*ef1a*) and ribosomal protein L13 alpha (*rp113a*), which were determined as the best reference genes using geNorm VBA applet for Microsoft Excel (Vandesompele *et al.* 2002).

Statistical analyses

Analysis of variance (ANOVA) was used to compare groups untrained, trained and recovered following Newman–Keuls multiple comparison test at a 5% significance level. Variation in proportion of muscle fibers was analyzed through *r x c* (2 x 2) table at a 5% significance level. When a significant difference was found, Marascuillo's method for multiple proportions comparison was applied (National Institute of Statistics—NIST, http://www.itl.nist.gov/div898/handbook). Hierarchical clustering of gene expression and heat maps were produced using PermutMatrix (http://www.atgc-montpellier.fr/permutmatrix) using McQuitty's method (Caraux *et al.* 2005).

Results

Growth analysis

Regarding growth analysis, we did not observe significant differences in fish length between the sample groups (Table 2). However, trained fish lost 26% of body weight when compared to untrained fish. Comparing the fish that had 72 hours of recovery after exercise with the untrained group, no significant difference was observed (Table 2). Finally, the condition factor of the trained group (7.5 ± 0.9) was lower when compared to untrained (10.8 ± 0.3) and recovered (9.4 ± 0.2) groups (Fig. 2A).

Lactate and protein content

In the present study, no significant differences were found in muscle lactate levels between the untrained (1.9 ± 0.3 mg mg^{-1}) and trained (1.6 ± 0.3 mg mg^{-1}) groups. Regarding the total protein concentration in the fish muscles of the different groups (Fig. 2B), a significant decreased was observed in trained group (0.14 ± 0.01 mg mg^{-1} protein) when compared to untrained (0.25 ± 0.02 mg mg^{-1} protein) and recovered (0.26 ± 0.02 mg mg^{-1} protein) groups.

Muscle histology

Muscle fibers analyses showed a significant increase in number of fibers of recovered (1,703 ± 73.6 fibers) when compared to the untrained (1,427.6 ± 73.7 fibers) and trained (1,307 ± 36.3 fibers) groups (Fig. 2C). Fibers distribution analysis showed significantly different profiles between the studied groups. Figure 3 shows that the relative proportion of fine fibers was significantly higher in trained group (61%) than in untrained (48%) and recovered (42%) groups.

Gene expression

Results of gene expression analyzes are compiled in Fig. 4. With regard to the IGF system genes, we did not observe any significant change in *igf1a* expression, but an induction of about 2 times of the *igf2a* gene was observed in the recovered group when compared to the trained group. We also observed a significant increase in *igf2b* gene expression of 1.8 and 2.2 times in the recovered group compared to the group trained and untrained, respectively. The *grna* gene was significantly induced in the recovered group when compared to the trained (2.7 times) and untrained (2.1 times) groups. However, a 40% decrease was identified in *pax7* gene expression in trained fish, when compared to untrained. The myogenic regulatory factors *mrf5* and *myod* were

significantly induced (4.4 and 2.4 times, respectively) in the recovered group when compared to the trained group. On the other hand, no change was detected in *myog* gene expression.

The hierarchical clustering of gene expression and the heat map are shown in Fig. 5. The induction of all genes of the trained (T) and recovered (R) groups was analyzed in relation to untrained group, grouping genes into three different clusters according to their expression profile. The first cluster group highlights a similarity between *grna* and *igf2b*. The second cluster is composed of all MRFs and *igf2a*, while the last one has only *igf1a* and *pax7*.

Discussion

Recent studies using counter current swimming exercise protocols have contributed to knowledge advance about skeletal muscle exercise adaptations, and has produced important information concerning fish muscle growth regulation (Palstra et al. 2010; Kirby and McCarthy 2013; Palstra et al. 2014). In this sense, the aim of this study was to evaluate the role of different IGFs in the zebrafish skeletal muscle regeneration after exercise. We used a 21 days training protocol with gradually increasing speed, wherein the maximum speed was 30 cm s^{-1} (10.5 BL s^{-1}). This training was considered of low intensity, while there was no difference in lactate concentration between trained and recovery fish.

The training protocol used in this work led to a significant decrease of 26% in weight, 29% in the condition factor and 44% in total protein content of trained fish when compared to untrained fish. Although there is no consensus about gain or loss of weight under exercise, several studies with fish found that moderate and light exercise can lead to weight loss (Davison and Goldspink 1978; Dougan 1993; McClelland et al. 2006) or feed efficiency reduction (Kiessling et al. 2011). Therefore, the exercise protocol applied in this study may have triggered a reduction in feed efficiency and an increased in catabolic state of the trained fish. Moreover, it is likely that trained fish have a higher metabolic demand and, thereby, the food quantity offered may not have been enough to provide the energy cost required by the exercise.

Regarding muscle fibers analysis, a significant difference in the fibers distribution was observed between trained and untrained fish. Greater number of thin fibers in trained fish was founded when compared to untrained. This result is in agreement with a decrease in skeletal muscle total protein content observed in trained

fish. Similar results were found in the distribution of red fiber banded wrasse (*Notolabrus fucicola*) trained at low intensity (2.3 BL s^{-1}) (Davison 1994). These results suggest that low-intensity swimming can cause a catabolic state in trained fish. On the other hand, the fish of recovery group were able to partially restore body weight and total protein content when compared to untrained fish. Beyond that, they showed a significant increase of 25% in the condition factor when compared to the trained group. These results are reflected in the muscle fibers distribution, where we observed a significant increase in fibers number and thick fibers proportion when comparing the trained group with recovered fish. In this work it became clear that skeletal muscle can restructure the muscle fibers and increase protein content after 72 hours of rest. However, a question arises: what is the mechanism involved in this muscle fibers regeneration process?

Due to IGF signaling paradigm act as a development regulator, growth and homeostasis (Edgar 2006), few studies have been conducted to evaluate the role of this pathway in different tissues regeneration (Chablais and Jazwinska 2010; Huang et al. 2013). To understand IGF system involvement in muscle regeneration we evaluated expression of *igf1a*, *igf2a* and *igf2b*, and also other major genes involved in the proliferation and differentiation processes of progenitor myogenic cells. Our results showed no significant difference in *igf1a* gene expression within any experimental group. Such ligand has been extensively characterized by showing mitogenic and anabolic effects in muscle cells (Glass 2003; Glass 2005; Duan et al. 2010.). However, there is no consensus about the role of IGF1 during fish myogenesis. Studies with rainbow trout revealed that IGF1 plays a relatively small effect on the differentiation process (Garikipati and Rodgers 2012a, b). On the other hand, Jimenez-Amilburu et al. (2013) have shown that IGF1 has a strong role in gilthead sea bream (*Sparus aurata*) muscle differentiation. In the same study, the authors demonstrated that IGF1 causes an increase in *mrf4* and *myog* expression, which have been associated with the final stages of muscle cells development, corresponding to differentiation (Rescan 2001; Holterman and Rudnicki 2005). In the present study, no change was observed in the levels of *myog* transcripts. Whereas a hyperplastic muscle fibers growth was observed in recovered fish without any alteration in *myog* and *igf1a* gene expression levels, our results suggest that these genes are not involved in the early stages of muscle regeneration in zebrafish.

IGF2, as well as IGF1, has been associated with fish postnatal muscle growth (Vong et al. 2003; Eppler et al 2010; Yuan et al. 2011). Gene expression results showed

an increase in transcript *igf2a* in the recovered group only when compared to the trained group. Though, a significant induction was observed in *igf2b* gene expression in the recovered group when compared to both trained and untrained. These results suggest greater involvement of the gene *igf2b* into muscle regenerative process after exercise than other zebrafish IGF system ligands. Furthermore, it was observed that the IGF2 induces *myf5* and *myod* expression fish muscle cells (Jimenez-Amilburu et al. 2013). MyoD and Myf5 are myogenic regulatory factors related to the maintenance of the myogenic lineage, hence, the proliferation of muscle cells (Rudnicki et al. 1993). Thereafter, increasing *myod* and *myf5* gene induction in the recovered group comparing to the trained group along with the increased *igf2a* and *igf2b* expression indicate that such genes are related to the initial cell proliferation process. In agreement with this hypothesis, Rius-Francino *et al.* (2011) observed myocyte proliferation in gilthead sea bream incubated with IGF2.

A similar result to *igf2b* was found for granulin (*grna*) expression. GrnA is recognized by its critical role in myogenic progenitor cells maintenance and is required for zebrafish muscle regeneration after injury (Li et al. 2013). Recent studies have shown that GrnA is involved in the muscle growth process through fibers fusion, thereby promoting a hypertrophic skeletal muscle growth (Hu et al. 2012; Li et al. 2013) This way, the clustering of *igf2b* and *grna* genes suggest that both are necessary in muscle regeneration. However, these growth factors seem to act differently in the growth of muscle tissue of fish. While *igf2b* promotes cell proliferation, *grna* would be involved with the initial hypertrophic growth. Therefore, we hypothesized that the increased expression of *igf2b* gene would be influencing the increase of fibers number in recovered fish, while the *grna* gene would be related to an increase in the proportion of thick fibers in recovered fish when compared to untrained.

Last but not least, the hierarchical cluster analysis grouped the genes into three distinct clusters. These results show that both *igf2a* and *igf2b* are involved in muscle regeneration process, but with different expression profiles, since they are in separate groups. Besides, the heat map shows that trained and recovered have distinct effects on gene expression. Therefore, we conclude that the joint action of growth factors IGF2A, IGF2B, GrnA and myogenic regulatory factors display a major response in the skeletal muscle regenerative process, reestablishing the zebrafish muscular structure after a catabolic state caused by exercise.

In conclusion, this study was the first to investigate the relationship of IGF genes with skeletal muscle regeneration after low intensity swimming exercise. Our results showed that both *igf2a* and *igf2b* are involved in this process. However, the results suggest a greater involvement of *igf2b* in muscle regeneration. Recently, our research group found that the *igf2b* is also involved in zebrafish muscle growth when stimulated by GH transgenesis (unpublished data). Thereby, our studies indicate that growth and regeneration use similar regulatory mechanisms and *igf2a* and *igf2b* play an important regulatory role in these processes, pointing out these growth factors as possible candidates for biomedical and aquaculture applications.

Acknowledgements

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Tables

Table 1. Primers used for real-time qPCR.

Genes	Primers sequence	Efficiency	Amplicon	GenBank
igf1a	F: 5'-CAGGCAAATCTCCACGATCTC-3'	100.0	60 bp	NM131825
	R: 5'-TTTGGTGTCCTGGGAATATCTGT-3'			
igf2a	F: 5'-TGCCAAGCCGGTGAAGTCGG-3'	100.0	51 bp	NM131433
	R: 5'-ACCTGCAGCGAGGTGGAGGAA-3'			
igf2b	F: 5'-ACCTGCCAAGTCAGAGAGGGACG-3'	100.0	63 bp	NM001001815
	R: 5'-GCGGGCATCACTGGAATGACCTT-3'			
grna	F: 5'-TGTTGCCCGTCTCCAAAG-3'	90.0	78 pb	NM001001949
	R: 5'-ACCCGGCCCACACTTATAC-3'			
pax7	F: 5'-GAACCGGATTCCCATTAGAAG -3'	87.8	69 pb	NM131332
	R: 5'-AAACTCCACCGAGTTGATTGA -3'			
myf5	F: 5'- TCCAATGGGCCTGCAAA -3'	90.5	52 pb	NM131576
	R: 5'-CGGCGGTCCACCGTACT-3'			
myod	F: 5'- GGAGCGAATTTCCACAGAGACT-3'	102.1	58 pb	NM131262
	R: 5'-GTGCCCCTCCGGTACTGA-3'			
myog	F: 5'-GGCCGCTACCTTGAGAGAGA-3'	92.3	59 pb	NM131006
	R: 5'- GAGCCTCAAAGGCCTCGTT-3'			
ef1α	F: 5'-GGGCAAGGGCTCCTTCAA-3'	100.0	54 bp	NM131263
	R: 5'-CGCTCGGCCTTCAGTTTG-3'			
rp113a	F: 5'-TCTGGAGGACTGTAAGAGGTATGC-3'	102.9	148 bp	NM212784
	R: 5'-AGACGCACAATCTTGAGAGCAG-3'			

Table 2. Growth parameters of adult zebrafish males.

All fish	**Weight (mg)**	**Length (mm)**
Untrained	311,3±18,7[a]	30,5±0,5
Trained	230,2±30,3[b]	29,3±0,3
Recovered	283,5±19,4[a,b]	31,4±0,6

Data are expressed as the mean ± SE. Different letters indicate significant differences ($p < 0.05$) among groups.

Figure legends

Figure 1: The swim-tunnel used in this study. TR: temperature regulator; R: water reservoir; P: centrifugal pump; OV: overflow valve; IV: inlet valve; SS: swimming section; E: fish entrance; N: screen. Arrows show current directions.

Figure 2: The body condition analysis. Condition factor **(A)**, skeletal muscle protein concentration **(B)** and total number of muscle fiber **(C)** in untrained, trained and recovered zebrafish with 6 months of age. Data are expressed as the mean ± SE. Different letters denote significant differences between treatment groups ($P < 0.05$).

Figure 3: Proportion of thin (≤18μm) and thick (>18μm) muscle fibers from untrained (A), trained (B) and recovered (C) zebrafish (*Danio rerio*). Differences were significantly different at $P<0.05$.

Figure 4: Relative gene expression comparing untrained, trained and recovered zebrafish (*Danio rerio*). The expression level of each gene was normalized by the elongation factor 1 alpha (*ef1α*) and ribosomal protein L13 alpha (*rp113a*) genes. Data are expressed as the mean ± SE ($n = 5$). Significant difference ($P < 0.05$) in gene expression level is denoted by different letters.

Figure 5: Hierarchical cluster analysis of genes expression. Heat map shows the hierarchical clustering of relative expression gene of trained and recovered zebrafish normalized by untrained zebrafish. Red refers to upregulation of gene expression, and green refers to downregulation of gene expression.

Figures

Figure 1

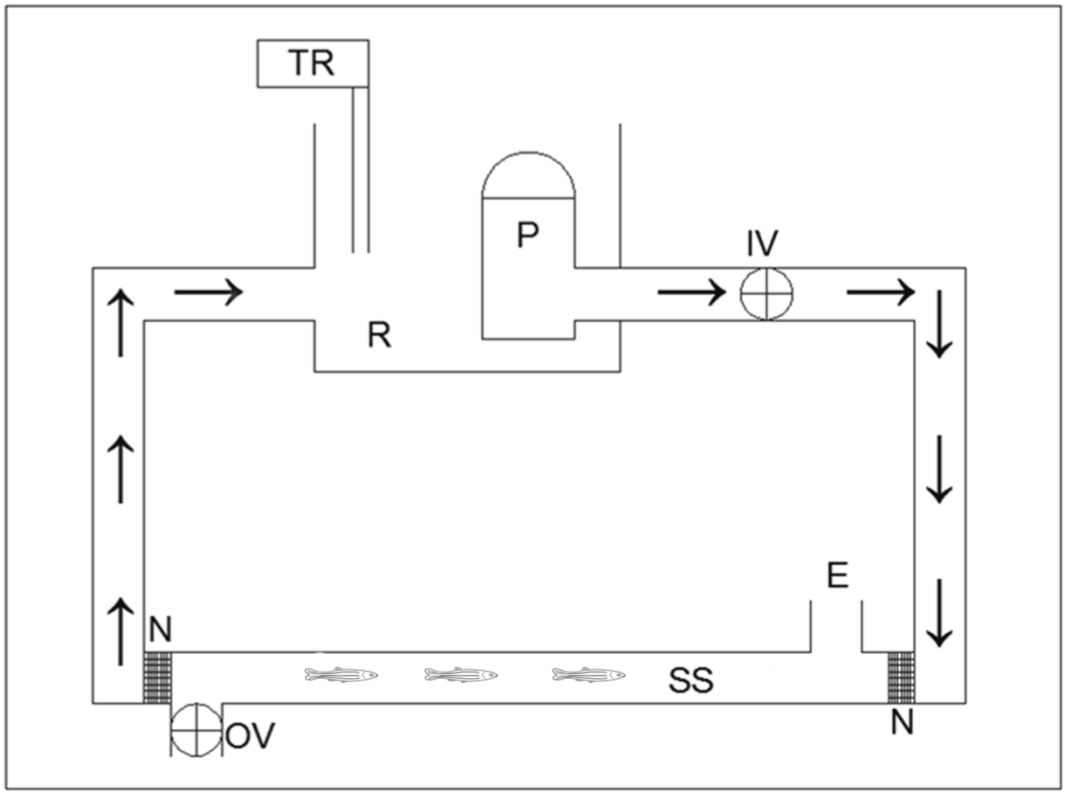

Figure 2

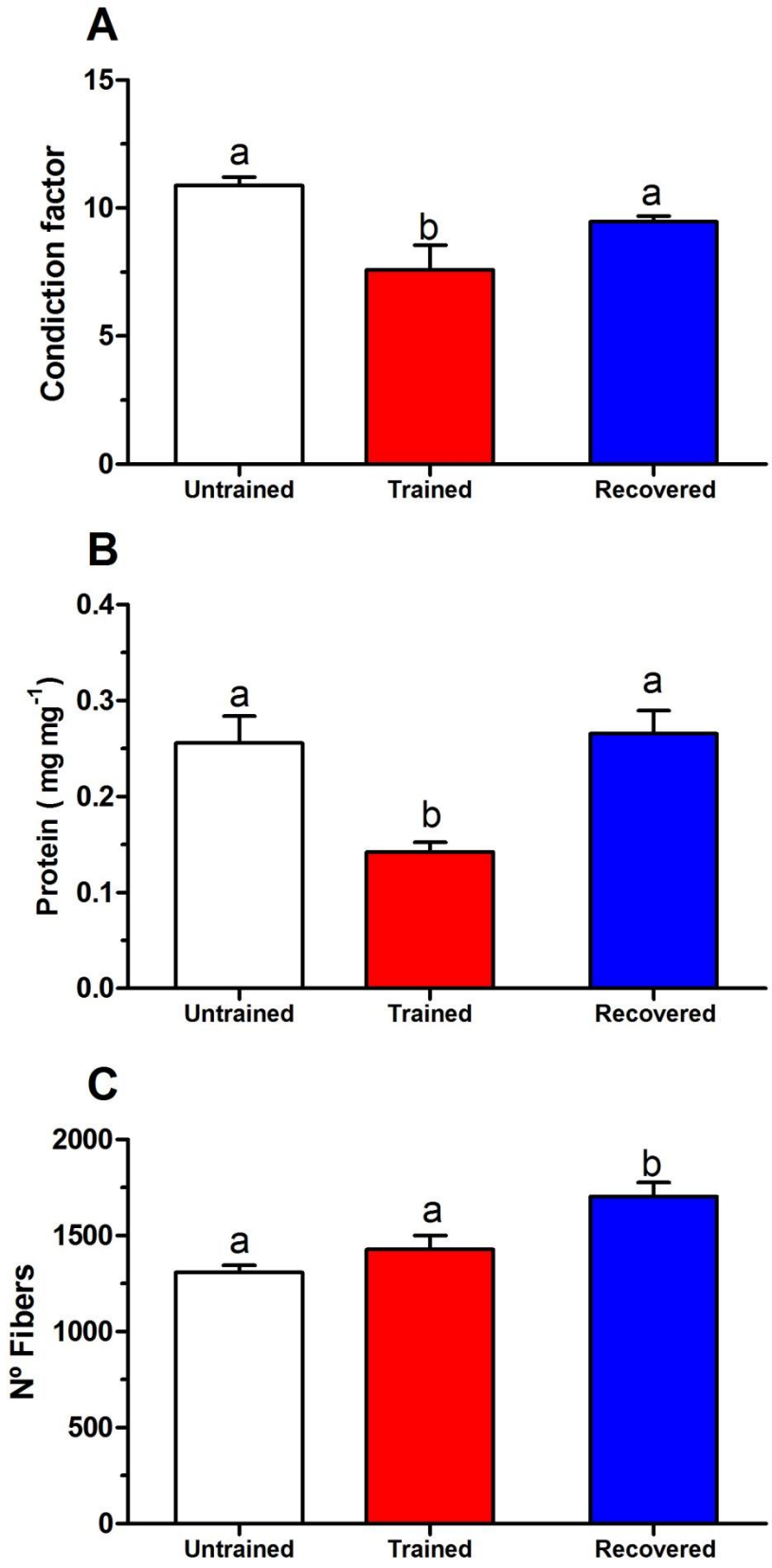
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Figure 3

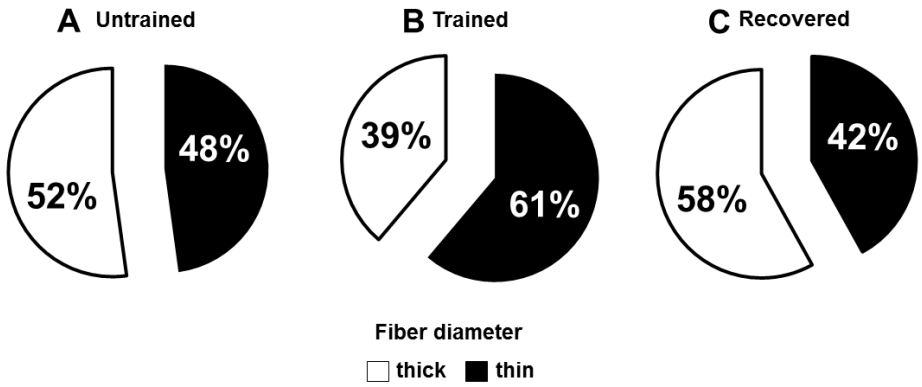


Figure 4

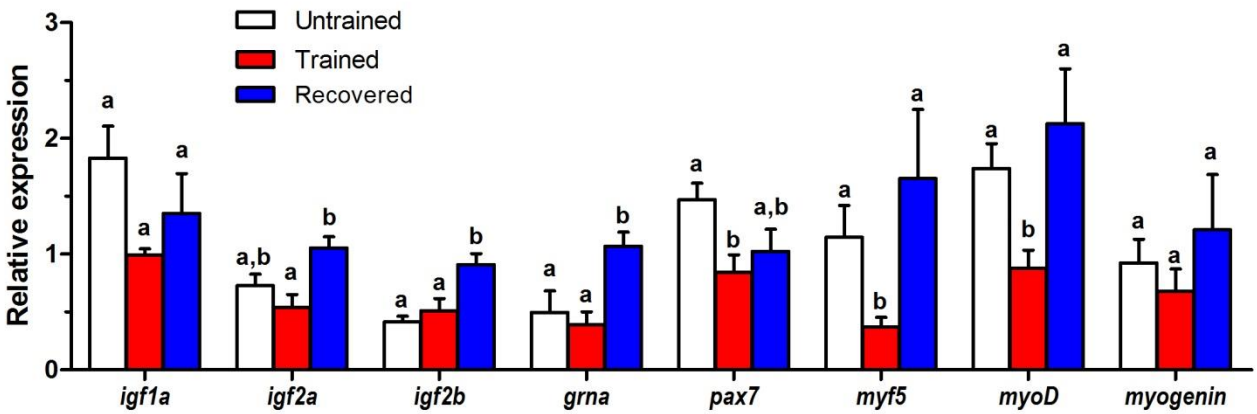
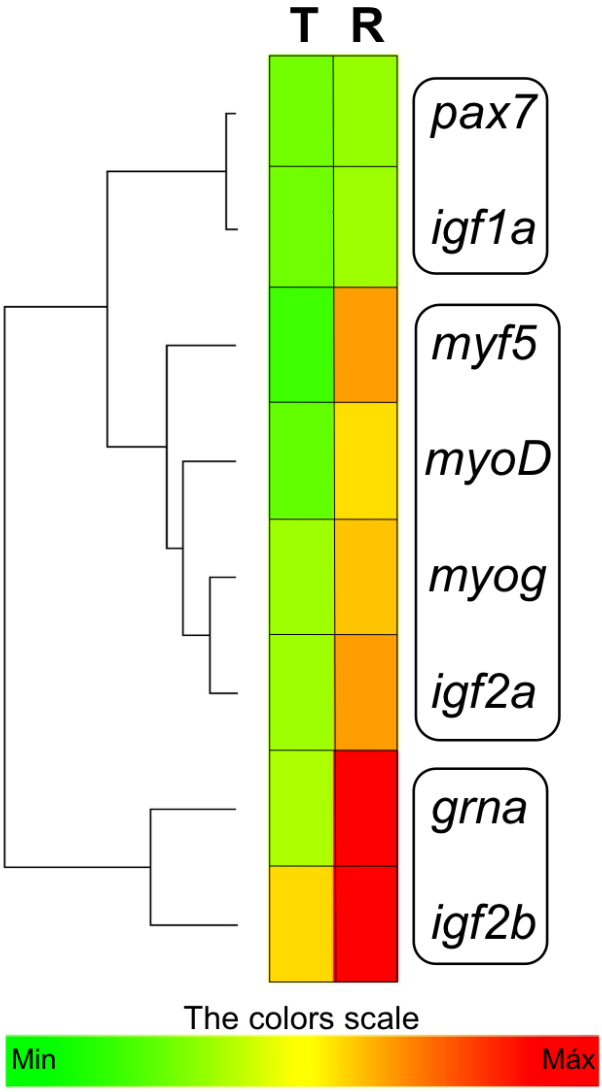


Figure 5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MANUSCRITO 3

GH INDIRECTLY ENHANCES THE REGENERATION OF TRANSGENIC ZEBRAFISH FINS THROUGH IGF2A AND IGF2B

Autores: Bruna Félix Nornberg, Daniela Volcan Almeida, Márcio Azevedo Figueiredo e Luis Fernando Marins

Manuscrito submetido ao periódico Transgenic Research

GH indirectly enhances the regeneration of transgenic zebrafish fins through IGF2a and IGF2b

Bruna Félix Nornberg, Daniela Volcan Almeida, Márcio Azevedo Figueiredo and Luis Fernando Marins*

Laboratório de Biologia Molecular, Instituto de Ciências Biológicas, Universidade Federal do Rio Grande – FURG, Rio Grande, RS – Brazil.

*Send correspondence to L.F. Marins. Universidade Federal do Rio Grande - FURG, Instituto de Ciências Biológicas, Av. Itália Km 8, CEP 96203-900, Rio Grande, RS - Brazil. E-mail: dqmluf@furg.br.

Abstract

The somatotropic axis, composed essentially of the growth hormone (GH) and insulin-like growth factors (IGFs), is the main regulator of somatic growth in vertebrates. However, these protein hormones are also involved in various other major physiological processes. Although the importance of IGFs in mechanisms involving tissue regeneration has already been established, little is known regarding the direct effects of GH in these processes. In this study, we used a transgenic zebrafish (*Danio rerio*) model, which overexpresses GH from the beta-actin constitutive promoter. The regenerative ability of the caudal fin was assessed after repeated amputations, as well as the expression of genes related to the GH/IGF axis. The results revealed that GH overexpression increased the regenerated area of the caudal fin in transgenic fish after the second amputation. Transgenic fish also presented a decrease in gene expression of the GH receptor (*ghrb*), in opposition to the increased expression of the IGF1 receptors (*igf1ra* and *igf1rb).* These results suggest that transgenic fish have a higher sensitivity to IGFs than to GH during fin regeneration. With respect to the different IGFs produced locally, a decrease in *igf1a* expression and a significant increase in both *igf2a* and *igf2b* expression was observed, suggesting that *igf1a* is not directly involved in fin regeneration. Overall, the results revealed that excess GH enhances fin regeneration in zebrafish through *igf2a* and *igf2b* expression, acting indirectly on this major physiological process.

Keywords: Fin regeneration; growth hormone (GH); insulin-like growth factor (IGF); transgenic zebrafish; gene expression.

Introduction

In mammals, the loss of a limb is irreversible. On the other hand, fish are able to regenerate their appendages completely after amputation. Zebrafish (*Danio rerio*) is an excellent model for regenerative studies because this teleost has the ability to regenerate many tissues and organs (Gemberling et al. 2013). The caudal fin of the zebrafish is one of the most-used tissues in regenerative studies because of its accessibility, simple architecture, and rapid regeneration (Iovine 2007; Wehner and Weidinger 2015). The regenerative ability of the fin depends directly on the interaction between the injured epithelium and the mesenchymal stem cells of the blastema (Poss 2010). However, cellular and molecular mechanisms controlling this phenomenon are very complex and have not yet been completely elucidated. Some signaling molecules have been directly related to this process, including insulin-like growth factors (IGFs) (Chablais and Jazwinska 2010).

IGFs are evolutionary preserved proteins, which activate an intercellular signaling cascade when binding to a tyrosine kinase-like receptor, promoting cellular proliferation, migration, and differentiation (Annunziata et al. 2011). The IGF system is widely known for mediating the actions of growth hormone (GH), thus performing key functions in neuroendocrine growth regulation in fish (Fuentes et al. 2013; Wood et al. 2005). It was recently reported that IGF signaling mediates the regeneration of the fin (Chablais and Jazwinska 2010) and cardiac tissue (Huang et al. 2013) in zebrafish. GH has also been related to the regeneration of several tissues, such as liver (Pennisi et al., 2004) and muscle (Reznick et al., 1996) in mammals, and inner ear cells (Schuck et al. 2011; Sun et al. 2011) in zebrafish. However, given the almost inseparable relationship between GH to IGFs, little is known concerning the individual participation of each component of the somatotropic axis in regenerative processes, and new study models are necessary to investigate this.

In order to study the effect of the GH/IGF axis on different physiological aspects of fish, we produced a transgenic zebrafish lineage (named F0104) that overexpresses GH from the beta-actin promoter (Figueiredo et al. 2007a). It has already been noted that excess GH induces an increase in IGF1 expression in the liver of transgenic fish of

the F0104 lineage (Figueiredo et al. 2007b). Considering the involvement of IGF signaling in fin regeneration in zebrafish, the objective of this study was to determine whether GH overexpression changes the regenerative ability of zebrafish through the IGF system.

Materials and Methods

Ethics Statement

All experiments involving animals were approved by the Ethics Committee for Animal Use at the Federal University of Rio Grande (proc. no. 23116.005269/2015-75), according to directives from Brazilian Guidelines for the Care and Use of Animals for Scientific and Educational Purposes – DBCA (RN 12, 2013 - CONCEA).

Fish

Transgenic (T) and non-transgenic (NT) adult zebrafish were obtained from crosses between NT females and hemizygous GH-transgenic males from the F0104 line, following a previously described protocol (Figueiredo et al. 2007a). For this study, T and NT zebrafish siblings were reared in a closed-circulation water system composed of 15L aquariums at 28°C, 14h light/10h dark photoperiod, fed with high-protein food (47.5%) twice a day, ad libitum. Water quality was monitored once a week, and temperature, pH, nitrogen compounds, and photoperiod were maintained according to zebrafish requirements (Westerfield 1995). When the fish reached eight months of age, 10 fish from the NT group and 17 fish from the T group were maintained at 28°C in separate tanks (one individual per tank) to perform the experiments. The fish were anesthetized in a 0.1 mg mL^{-1} tricaine solution and amputated using a razor blade.

Fin Area Measurement

In order to assess the regenerative ability of transgenic GH animals, T and NT fish underwent three partial amputations of their caudal fins, and the tissue removed after each amputation was used for molecular analyses (Fig. 1). Caudal fins were photographed 72 h after the first two amputations (72 hpa) using a SC30 digital camera (Olympus, Japan) coupled with a SZX16 stereoscopic microscope (Olympus, Japan). The regeneration area was normalized by the fin width (Varga et al. 2014). To quantify the regenerated area, we used the Image J software.

Gene Expression

The tissues were collected immediately after the initial amputation (R0) and 72 h after successive amputations (R1 and R2). Total RNA was isolated using TRIzol Reagent (Invitrogen, Brazil) and used as a target for cDNA synthesis via a High Capacity Reverse Transcription kit (Applied Biosystems, Brazil) following the manufacturer's instructions.

Gene expression was analyzed using quantitative Real-Time PCR (RT-qPCR). Each sample (n = 6) was analyzed in duplicate. Specific primers for each gene (Table 1) were designed using the Primer-BLAST tool from GenBank (http://www.ncbi.nlm.nih.gov). RT-qPCR was performed in a 7500 Real Time System (Applied Biosystems, Brazil) using SYBR Green PCR Master MixTM (Invitrogen, Brazil) according to the manufacturer's protocol. Five-point standard curves of a five-fold dilution series from pooled cDNA were used for the calculation of PCR efficiency. PCR conditions were 50°C/2 min, 95°C/2 min, followed by 40 cycles at 95°C/15 s and 60°C/30 s. Four candidate reference genes (elongation factor 1 alpha, *ef1α*; beta-2-microglobulin, *b2m*; beta-actin, *actb1*; and ribosomal protein L13 alpha, *rp113a*) were tested using the geNorm VBA applet for Microsoft Excel (Vandesompele et al., 2002). Consequently, we calculated a normalization factor based on the expression levels of the best-performing housekeeping genes. Among the candidates, *ef1α*, *actb1*, and *rp113a* were selected as reference genes for the relative gene expression analysis.

Statistical analysis

Fin regeneration and relative expression between treatments were compared using a two-way analysis of variance (ANOVA), followed by the Newman–Keuls multiple comparison test ($p < 0.05$). ANOVA assumptions (normality and homogeneity of variance) were verified prior to testing. All data were expressed as the mean ± standard error (SE).

Results and Discussion

Transgenic models have been considered as important tools for the elucidation of the molecular mechanisms involved in regeneration (Azevedo et al. 2011; Singh et al. 2012; Tryon and Johnson 2014). In the present study, we used a transgenic zebrafish to assess the effect of GH overexpression on fin regeneration. Our results revealed no significant difference in the regenerated area of the fin after the first (R1) and the

second amputation (R2) in NT fish (0.42 ± 0.02 and 0.48 ± 0.02 mm2, respectively) (Fig. 2a). Indeed, it has been reported that successive amputations do not affect the regenerative ability of the caudal fin of zebrafish (Azevedo et al. 2011). However, a different result was observed in transgenic fish (R1, 0.40 ± 0.01 and R2, 0.60 ± 0.01 mm2). Interestingly, we identified a significant (25%) increase in the regenerated area of T fish caudal fins with respect to that in NT fish after the second amputation. In this context, a question arises: how does GH act on the regenerating tissue?

One method to analyze the function of a hormone is to quantify the expression of its receptor on the target tissue. We analyzed the GH receptor (GHR) expression in T and NT animal fins (Fig. 3a). No significant change was observed in the expression of this gene in NT fish. However, a 2.9-fold increase in *ghrb* expression was observed in the fin of T fish immediately after the first amputation (R0), representing the status of the tissue before the regeneration. Therefore, this result indicated that excess GH affected the fin in T fish and probably activated its main JAK/STAT signaling pathway, which culminates in the induction of IGF1 expression (Lanning and Carter-Su 2006). Supporting this result, an approximately two-fold increase was observed in *igf1a* transcripts in T fish over those in NT at R0 (Fig. 4a). These results indicated that the classic GH action pathway is naturally activated in the fins of transgenic animals. However, after the two amputations, the regenerated tissue exhibited a decrease of approximately 70% in *ghrb* expression in T fish compared to its expression at R0 (Fig. 3a). This result suggests that GH may not be directly related to the increase in the regeneration in T animals. On the other hand, it has already been determined that *igf1ra* and *igf1rb* are expressed in the caudal fin and are necessary for regeneration (Chablais and Jazwinska 2010). Thus, we assessed gene expression of these receptors and identified an increase in *igf1ra* (2.0-fold in R1 and R2) and *igf1rb* (3.7- and 2.5-fold in R1 and R2, respectively) transcripts for the transgenic group only (Fig. 3b, c). Consequently, the decrease in *ghrb* expression and increase in igf1ra and b expression suggest that transgenic animals have a higher sensitivity to IGFs during the regeneration process.

In zebrafish, unlike mammals, four different genes of the IGF system (*igf1a*, *igf1b*, *igf2a* and *igf2b*) have been identified and characterized (Zou et al. 2009). In order to assess the role of the different IGFs in the increased regeneration in T fish, the expression of *igf1a*, *igf2a*, and *igf2b* genes (Fig. 4) was analyzed in both groups. The analysis of igf1a gene expression (Fig. 4a) revealed a significant decrease in gene

expression at R1 and R2 in both NT (67% and 61%, respectively) and T (63% and 79%, respectively) groups when compared to that at R0. Results for *igf1a* gene expression indicated that this ligand is not directly related to the fin regeneration process. Expression of *igf2a* (Fig. 4b) was not significantly different among amputation times in NT. However, T fish exhibited a significant 1.5-fold increase in the expression of this gene at R2 over that at R0. As to *igf2b* expression, there was a significant increase in both groups at subsequent amputations over the first amputation. NT fish had a 1.8- and 2.5-fold increase (R1 and R2, respectively), whereas T fish had a 4.8-fold increase in R1 and a 2.7-fold increase in R2. Chablais and Jazwinska (2010) showed that the amputation of the caudal fin in zebrafish induces the expression of the IGF2b ligand in the blastema, leading to the activation of IGF signaling, specifically in cells adjacent to the apical epithelium. This mechanism represents a crucial step in regenerating this appendage. In this context, our results indicated that both *igf2b* and *igf2a* might be related to fin regeneration in transgenic animals. Therefore, the peak in *igf2b* expression in R1, together with the increase in receptor transcripts, is a plausible explanation for the increase in the regeneration area of transgenic animals.

This is the first study to use a GH-transgenic fish model to investigate the involvement of excess hormone in the regenerative process. In this study, we showed that GH enhances the regeneration of the zebrafish caudal fin after second amputation. However, our results revealed that GH acts indirectly on this process through the IGF system. Nonetheless, the antagonistic response observed in the expression of IGF receptors when compared to that of GHR raises a question: how does GH influence the increase in *igf2a* and b expression, as well as that in *igf1ra* and *b* receptors in T fish? In a parallel work of our group it was observed that excess GH induces an increase in *igf2a* and *igf2b* expression in the liver of the F0104 line (unpublished data). Thus, we considered the possibility that circulating IGF2a and IGF2b may induce their auto-regulation in T fins. This mechanism has been previously observed in the differentiation of muscle cells in mammals (Ren et al. 2008). Finally, we highlight that excess GH may exert a pro-regenerative effect through the IGF system in fish. In addition, we reveal that the relevance of the GH/IGF axis extends beyond its classic actions on somatic growth and energy metabolism.

Acknowledgements

This work was financially supported by Brazilian CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Proc. No. 453966/2014-4). Luis Fernando Marins is a research fellow from CNPq (Proc. No. 304675/2011-3).

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Table

Table 1. Analyzed genes and primers used for real-time qPCR.

Genes	Primers sequence	Efficiency	Amplicon	GenBank
Igf1a	F: 5'-CAGGCAAATCTCCACGATCTC-3'	100.0	60 bp	NM131825
	R: 5'-TTTGGTGTCCTGGGAATATCTGT-3'			
Igf2a	F: 5'-TGCCAAGCCGGTGAAGTCGG-3'	100.0	51 bp	NM131433
	R: 5'-ACCTGCAGCGAGGTGGAGGAA-3'			
Igf2b	F: 5'-ACCTGCCAAGTCAGAGAGGGACG-3'	100.0	63 bp	NM001001815
	R: 5'-GCGGGCATCACTGGAATGACCTT-3'			
Igf1ra	F: 5'-GATCCAAAGAGCAGGGCTCC-3'	100.0	88 bp	NM152968
	R: 5'-GCCATCCCATCCGCTATCTC-3'			
Igf1rb	F: 5'-GGTCTAGCAAACAGAGGCGA-3'	101.8	132 bp	NM152969
	R: 5'-CCAGCCGCTTGAAATTACCG-3'			
Ghrb	F: 5'- GAACTCAGAGTCCGGGCAAA-3'	99,5	117 pb	NM001111081
	R: 5'- AAAGACCAGCACAGCCGTAA-3'			
Ef1α	F: 5'-GGGCAAGGGCTCCTTCAA-3'	100.0	54 bp	NM131263
	R: 5'-CGCTCGGCCTTCAGTTTG-3'			
Rp113a	F: 5'-TCTGGAGGACTGTAAGAGGTATGC-3'	102.9	148 bp	NM212784
	R: 5'-AGACGCACAATCTTGAGAGCAG-3'			
B2m	F: 5'-GCCTTCACCCCAGAGAAAGG-3'	100.5	101 bp	NM131163
	R: 5'-GCGGTTGGGATTTACATGTTG-3'			
Actb1	F: 5'-GCTGTTTTCCCCTCCATTGTT-3'	100.5	60 bp	NM131031
	R: 5'-TCCCATGCCAACCATCACT-3'			

Figure legends

Fig. 1. Outline of the consecutive caudal fin amputations. The caudal fin of the animals (T and NT) was partially amputated three times (C1, C2, and C3). Seventy-two h after each amputation (72 hpa), the animals were photographed in order to assess the regenerated area. R0: Tissue removed immediately after the initial amputation; R1: Tissue removed 72 h after the first amputation; R2: Tissue removed 72 h after the second amputation.

Fig. 2. Area regenerated after consecutive amputations. (a) Comparison of the regenerated area of the caudal fins of transgenic (T) and non-transgenic (NT) zebrafish 72 h after the first (R1) and second (R2) amputations. (b) The same caudal fin 72 h after the first (R1) and second (R2) amputation of T and NT.

Fig. 3. Relative gene expression of *ghrb* (a), *igfr1a* (b), and *igfr1b* (c) in transgenic (T) and non-transgenic (NT) zebrafish fin. R0: fin immediately after the initial amputation; R1: fin regenerated 72 h after first amputation; R2: fin regenerated 72 h after second amputation. Data are expressed as the mean ± SE (n = 6). Different letters indicate significant differences ($p < 0.05$) between groups.

Fig. 4. Relative gene expression of *igf1a* (a), *igf2a* (b) and *igf2b* (c) in transgenic (T) and non-transgenic (NT) zebrafish fin. R0: fin immediately after the initial amputation; R1: fin regenerated 72 h after first amputation; R2: fin regenerated 72 h after second amputation. Data are expressed as the mean ± SE (n = 6). Different letters indicate significant differences ($p < 0.05$) between groups.

Figures

Figure 1

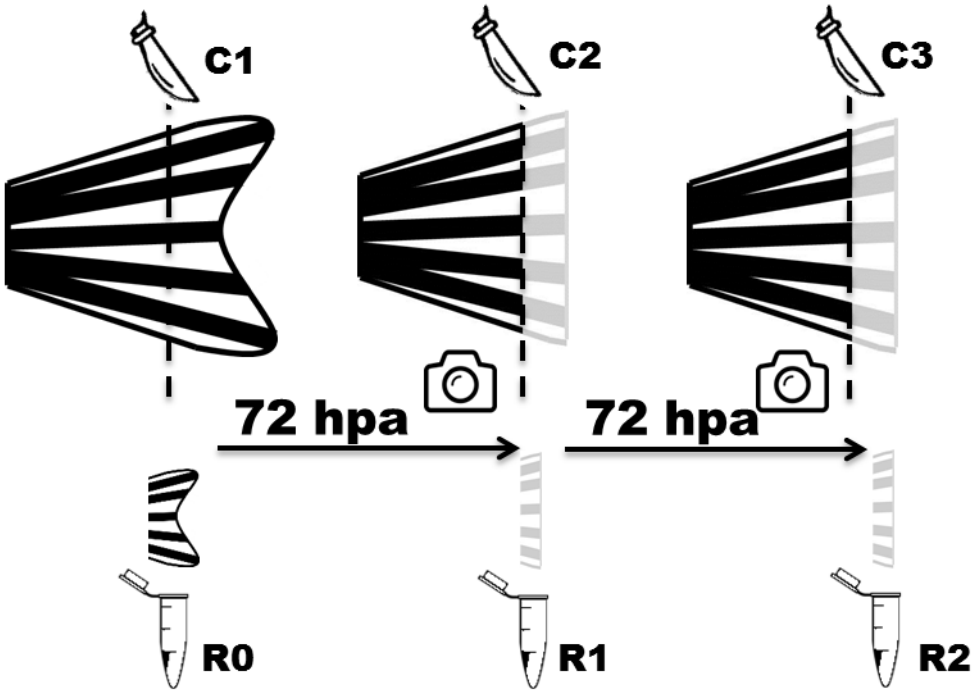


Figure 2

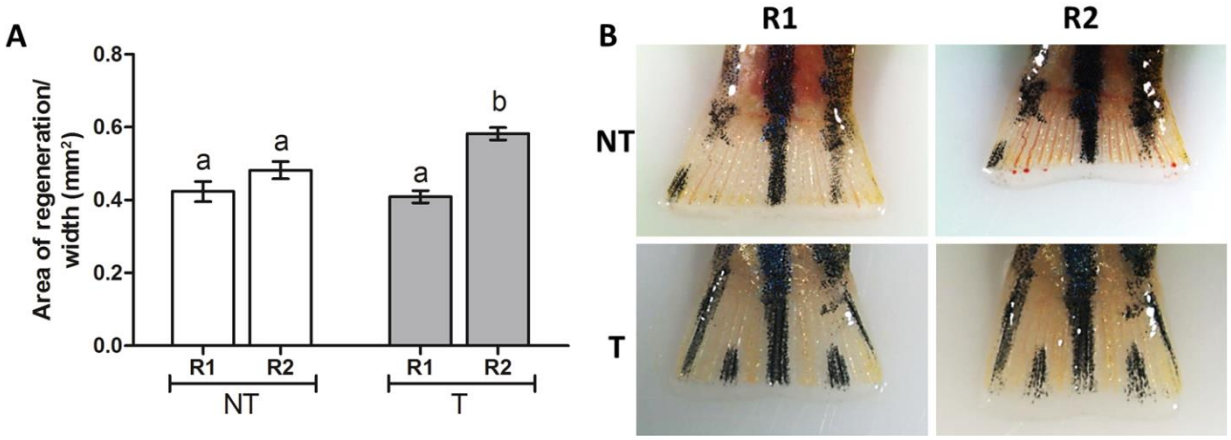


Figure 3

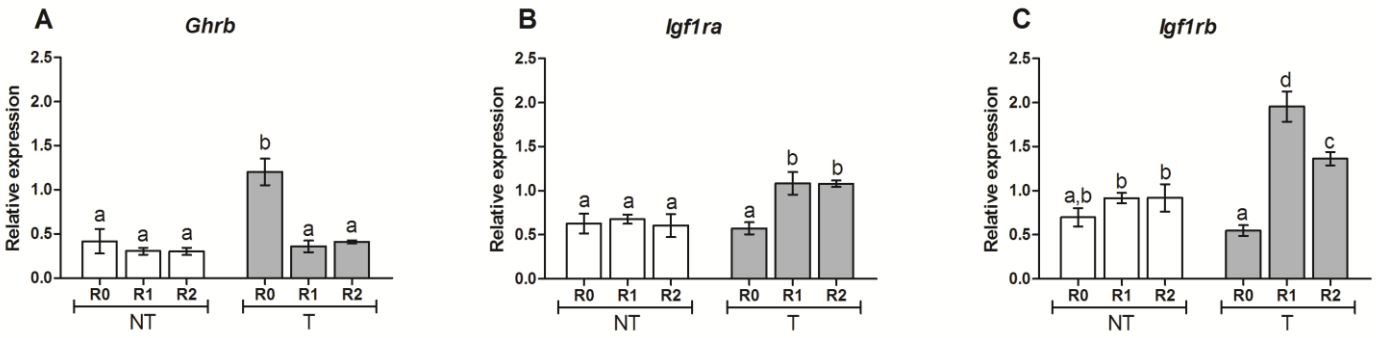
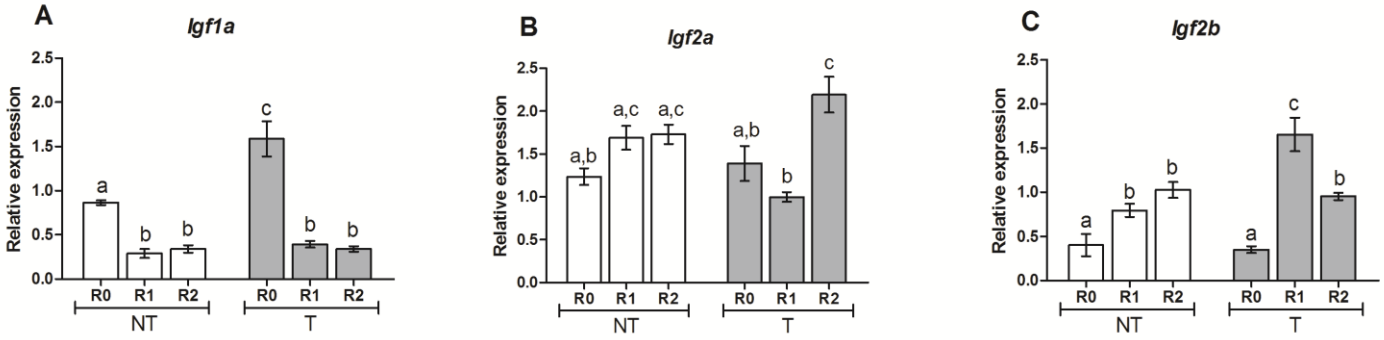


Figure 4



DISCUSSÃO GERAL

O mecanismo regulatório do crescimento em peixes via eixo GH/IGF tem sido bem estudado (Fuentes *et al.* 2013a; Yan Xiang *et al.* 2015). Grande parte dos avanços no conhecimento deste eixo em teleósteos é devido a estudos utilizando linhagens geneticamente modificadas para o GH (Devlin *et al.* 1994; Rahman *et al.* 1998; Nam *et al.* 2001; Figueiredo *et al.* 2007b). Entretanto, questões sobre qual a importância dos IGFs secretados pelo fígado (IGFs endócrinos) versus IGFs autócrino/parácrino produzidos no músculo sobre o crescimento muscular, ainda não foram totalmente esclarecidas. Além disso, não há um consenso se o GH é um potente regulador dos IGFs na musculatura. Kuradomi *et al.* (2011) não encontraram alteração nos transcritos de IGF1 no músculo de zebrafish GH transgênico (linhagem F0104). Além disso, estudos recentes tem demonstrado que a expressão de IGF1e IGF2 pode ser fortemente controlada por nutrientes e pelos próprios IGFs (Ren *et al.* 2008; Fuentes *et al.* 2011, 2013b). Neste contexto, no primeiro capítulo desta tese foi avaliado o perfil de expressão dos diferentes IGF no fígado e músculo de zebrafish GH-transgênico. Os resultados mostraram que o GH está ativando a via clássica de sinalização (JAK/STAT), aumentando o número de transcritos de três IGFs distintos no fígado. Por outro lado, o músculo não respondeu ao excesso de GH da mesma forma. Apenas a expressão do *igf2b* foi induzida no músculo, o que sugere uma importância deste ligante no processo de crescimento muscular de zebrafish. Corroborando com este resultado, Vong *et al.* (2003) encontraram um aumento apenas nos transcritos de *igf2* no músculo de carpas comuns injetadas com GH. Além disso, no músculo de tilápias transgênicas para o GH foi observado uma indução de ambos *igf1* e *igf2* (Eppler *et al.* 2010). Além do aumento da expressão de *igf2b* no músculo, também foi evidenciado um aumento na expressão da *igfbp5b* no músculo dos animais transgênicos. A IGFBP5 tem sido relacionada com a autoregulação da expressão do IGF2 em células musculares de mamíferos (Ren *et al.* 2008). Portanto, na linhagem F0104 a IGBP5 pode estar envolvida com o aumento da disponibilidade de IGF2 ao IGF1R, conduzindo a ativação da via de sinalização PI3k/Akt/mTOR (Fig. 10). Este mecanismo pode ser responsável por promover a hipertrofia muscular observada nos animais transgênicos. Assim, estes resultados chamam a atenção para a importância autócrina/parácrina do IGF2 no crescimento muscular de teleósteos, entretanto o conhecimento sobre as ações deste ligante sobre musculatura de teleósteos não está completamente elucidado.

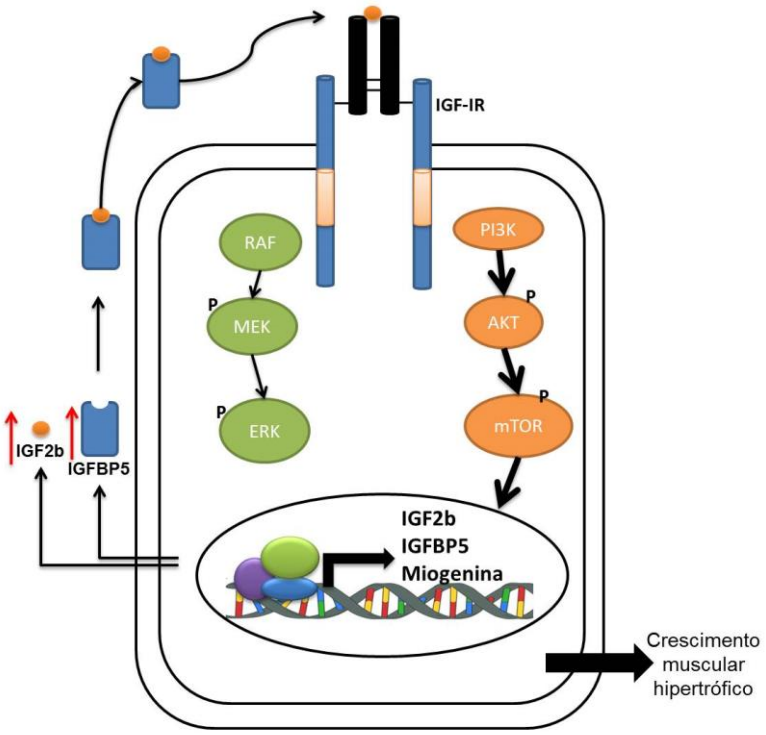


Figura 10: Hipótese para o crescimento hipertrófico muscular dos animais GH-transgênicos. O esquema acima representa a diferenciação das células musculares através da síntese de IGF2b e IGFBP5 durante a miogênese nos animais transgênicos. A IGFBP5 é induzida nas fases iniciais de miogênese e está localizada na superfície da célula. A IGFBP5 associa-se ao IGF2b e conduz este ligante as proximidades do receptor do IGF1R, aumentando assim a atividade de sinalização deste receptor. A ligação do IGF2b ao IGF1R ativa fortemente a PI3k-Akt-mTOR levando a um aumento na expressão do gene de IGF2b e portanto, sua auto-regulação. A sinalização da PI3k-Akt-mTOR também pode levar ao aumento de MRFs, os animais transgênicos possuem um aumento de miogenina o que pode estar relacionado com o crescimento muscular hipertrófico.

A musculatura esquelética possui uma grande capacidade para regenerar em resposta ao trauma, o que pode ocorrer agudamente em resposta a toxinas, exercício ou mais cronicamente em caso de distrofia muscular. A capacidade regenerativa da musculatura depende da proliferação e diferenciação de células progenitoras miogênicas, que são responsáveis pelo crescimento hipertrófico e hiperplásico (Koumans & Akster 1995; Johansen & Overturf 2005; Buckingham 2006). O sistema IGF é um dos principais reguladores deste processo (Johnston 2006). No segundo capítulo desta tese foi avaliado o envolvimento dos diferentes IGFs na regeneração da musculatura após um estado catabólico gerado pelo exercício de natação. Foi observado que ambos, IGF2a e IGF2b estão envolvidos com a regeneração da musculatura de zebrafish, porém os resultados indicam um maior envolvimento do IGF2b neste processo.

Já foi previamente observado que a hiperplasia e hipertrofia da musculatura é regulada pelos MRFs, MyoD, Myf5, MRF4 e miogenina (Perry & Rudnicki 2000).

Neste trabalho foi observado um crescimento hiperplásico dos animais recuperados após treinamento juntamente com o aumento nos níveis de transcrito de MyoD e Myf5. Estes resultados indicam que a proliferação celular pode estar relacionada com o aumento destes MRFs. Recentemente, Jiménez-Amilburu *et al.* (2013) observou que o IGF2 pode induzir um aumento de MyoD e Myf5 em células musculares de dourado, o que sugere que IGF2a e b possam estar mais envolvidos com os processos iniciais da miogênese durante a regeneração de zebrafish. Recentemente, Palstra e colaboradores (2014) observaram um crescimento hipertrófico da musculatura esquelética de zebrafish após exercícios de natação. Interessantemente, os resultados da análise de transcriptoma mostraram que apenas o IGF2 estava induzido no músculo dos animais treinados (Palstra *et al.* 2014). Dentro deste contexto, o conjunto de resultados apresentados até o momento mostra que os IGF2 são fundamentais para que ocorra o crescimento e a regeneração da musculatura em zebrafish, porém mais estudos são necessários para avaliar o papel deste ligante nos diferentes eventos da miogênese.

Os resultados obtidos sobre o envolvimento autócriono/parácrino do IGF2b no crescimento e regeneração muscular chamaram nossa atenção para este ligante que ainda não tem suas funções fisiológicas bem claras. Estudos recentes mostraram que além de estar envolvido com o desenvolvimento (White *et al.* 2009), o IGF2b também está relacionado com a regeneração de cardiomiócitos (Huang *et al.* 2013) e da nadadeira de zebrafish (Chablais & Jazwinska 2010). Neste sentido, o último capítulo desta tese teve como objetivo avaliar se o excesso de GH circulante é capaz de potencializar a regeneração da nadadeira caudal de zebrafish da linhagem F0104 através da sinalização do IGF. Foi demonstrado que o GH potencializa a regeneração de zebrafish após a segunda amputação e que ambos, IGF2a e IGF2b estão relacionados com esta resposta. Assim, os resultados apresentados aqui mostram que a relevância do eixo GH/IGF ultrapassa as ações clássicas sobre o crescimento somático e metabolismo energético e chamam a atenção para uma maior importância dos IGF2 sobre a regeneração.

A maioria dos estudos relaciona o IGF1 como o principal mediador das ações do GH no músculo, sendo este ligante reconhecido como um agente hipertrófico (Glass 2003; Clemmons 2009). Corroborando com esta afirmação, a geração de camundongos com expressão muscular de uma isoforma de IGF1 tem demonstrado desenvolvimento muscular normal e crescimento hipertrófico (Musarò *et al.* 2001). Entretanto, Li *et al.* (2014) produziram uma carpa (*Carassius auratus*) superexpressando IGF1 de zebrafish

no músculo esquelético e, interessantemente, encontraram uma queda no peso corporal e um crescimento hiperplásico da musculatura nos animais transgênicos (Li *et al.* 2014). Esta observação sugere que o IGF1 autócrino/parácrino pode não estar funcionando como um agente hipertrófico para esta espécie. Neste contexto, os resultados desta tese evidenciam que os IGF2 exercem um papel regulador importante sobre os processos de regeneração e crescimento de zebrafish (Fig. 11), o que chama atenção para possíveis aplicações destes fatores em estudos envolvendo as ciências biomédicas e para a aquicultura.

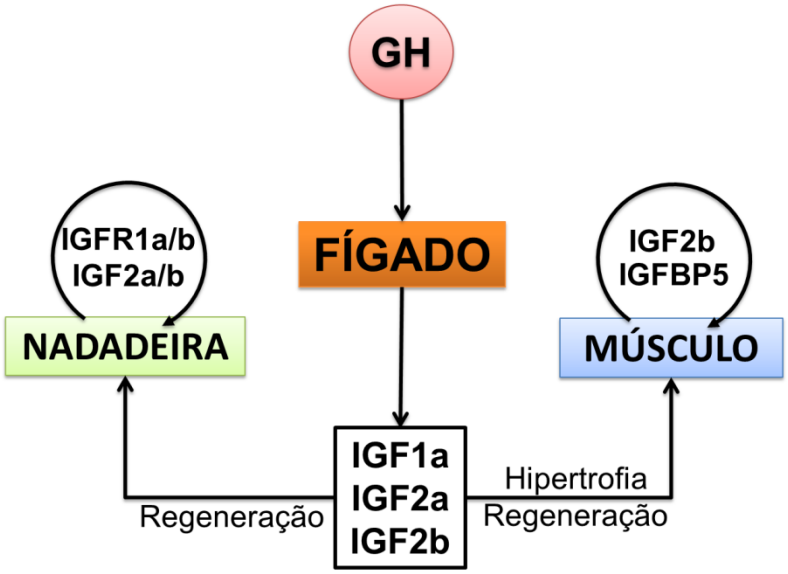


Figura 11: Proposta da ação do eixo GH/IGF sobre a musculatura e nadadeira de zebrafish. O GH promove um aumento na expressão de IGF1a, IGF2a e IGF2b no fígado através da via JAK/STAT. O aumento de IGF2 circulante pode induzir sua auto regulação nos tecidos da nadadeira e na musculatura promovendo regeneração de ambos os tecidos e hipertrofia da musculatura.

PERSPECTIVAS

Os resultados da presente tese contribuem para o conhecimento da função dos diferentes IGFs sobre o crescimento muscular e regeneração de zebrafish. Entretanto, ainda há questões importantes para serem esclarecidas sobre este tema. Dessa forma, como continuação deste estudo pretende-se:

- Produzir linhagens de zebrafish transgênicos com superexpressão dos diferentes IGF2 no músculo;
- Produzir uma linhagem que superexpressa o IGFR1 de forma músculo específica;
- Produzir linhagens celulares estabelecidas de musculatura de zebrafish para compreender melhor a regulação das diferentes vias de sinalização frente o crescimento hipertrófico e hiperplásico;

BIBLIOGRAFIA GERAL

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ANEXOS

Anexo 1- Protocolo de aprovação do CEUA referente aos experimentos do artigo 2

COMISSÃO DE ÉTICA EM USO ANIMAL
Universidade Federal do Rio Grande
Pró-Reitoria de Pesquisa e Pós-Graduação - PROPESP
ceua@furg.br http://www.propesp.furg.br

CEUA

CEUA - FURG FL.....

CERTIFICADO Nº P053/2015

Certificamos que o projeto intitulado "**Adaptações fisiológicas da musculatura esquelética de *zebrafish* (*Danio rerio*) em resposta ao treinamento de natação**", protocolo nº 23116.005403/2015-38, sob a responsabilidade de Luis Fernando Marins - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi APROVADO pela COMISSÃO DE ÉTICA EM USO ANIMAL DA UNIVERSIDADE FEDERAL DO RIO GRANDE (CEUA-FURG), em reunião de 30/09/2015 (Ata 013/2015).

A CEUA lembra aos pesquisadores que qualquer alteração no protocolo experimental ou na equipe deve ser encaminhada à comissão para avaliação e aprovação. Um relatório final deve ser enviado à CEUA no término da vigência do seu projeto.

CEUA Nº	Pq026/2015
VIGÊNCIA DO PROJETO	31/12/2015
ESPÉCIE/ LINHAGEM	*Danio rerio*
NÚMERO DE ANIMAIS	90
PESO/ IDADE	300 mg/ adultos
SEXO	Macho
ORIGEM	Biotério Aquático do ICB - FURG
ENVIO DO RELATÓRIO FINAL	31/01/2016

Rio Grande, 13 de outubro de 2015.

Med. Vet. Alice T. Meirelles Leite
Coordenadora da CEUA-FURG

Controle da CEUA - Parecer(es) prévio(s) relacionado(s) ao processo: P046/2015 Página 1/1

Anexo 2- Protocolo de aprovação do CEUA referente aos experimentos do artigo 3

COMISSÃO DE ÉTICA EM USO ANIMAL
Universidade Federal do Rio Grande
Pró-Reitoria de Pesquisa e Pós-Graduação - PROPESP
ceua@furg.br http://www.propesp.furg.br

CEUA

CEUA - FURG Fl.

CERTIFICADO Nº P052/2015

Certificamos que o projeto intitulado "**Efeito do excesso de GH sobre a regeneração da nadadeira caudal de *zebrafish***", protocolo nº 23116.005269/2015-75, sob a responsabilidade de Luis Fernando Marins - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi APROVADO pela COMISSÃO DE ÉTICA EM USO ANIMAL DA UNIVERSIDADE FEDERAL DO RIO GRANDE (CEUA-FURG), em reunião de 30/09/2015 (Ata 013/2015).

A CEUA lembra aos pesquisadores que qualquer alteração no protocolo experimental ou na equipe deve ser encaminhada à comissão para avaliação e aprovação. Um relatório final deve ser enviado à CEUA no término da vigência do seu projeto.

CEUA Nº	Pq025/2015
VIGÊNCIA DO PROJETO	31/01/2016
ESPÉCIE/ LINHAGEM	*Danio rerio* F0104GH-transgênico e não transgênico
NÚMERO DE ANIMAIS	48 (24 de cada linhagem)
PESO/ IDADE	500 mg/ adultos (transgênicos) 300 mg/ adultos (não transgênicos)
SEXO	Macho
ORIGEM	Laboratório de Transgênicos do ICB/FURG
ENVIO DO RELATÓRIO FINAL	Fevereiro de 2016

Rio Grande, 13 de outubro de 2015.

Med. Vet. Alice T. Meirelles Leite
Coordenadora da CEUA-FURG

Controle da CEUA - Parecer(es) prévio(s) relacionado(s) ao processo: P045/2015 Página 1/1