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O POTENCIAL DAS CÉLULAS INTESTINAIS COMO FONTE DE CÉLULAS PROGENITORAS PANCREÁTICAS

DISSERTAÇÃO DE MESTRADO

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Santa Maria, RS, Brasil

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O POTENCIAL DAS CÉLULAS INTESTINAIS COMO FONTE DE CÉLULAS PROGENITORAS PANCREÁTICAS

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Orientador: Prof. Paulo Bayard Dias Gonçalves

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elaborada por
Werner Giehl Glanzner

como requisito parcial para obtenção do grau de
Mestre em Medicina Veterinária

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RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

O POTENCIAL DAS CÉLULAS INTESTINAIS COMO FONTE DE CÉLULAS PROGENITORAS PANCREÁTICAS

AUTOR: WERNER GIEHL GLANZNER
ORIENTADOR: PAULO BAYARD DIAS GONÇALVES
Data e Local da Defesa: Santa Maria, 24 de fevereiro de 2012.

O objetivo do presente trabalho foi estabelecer um cultivo primário de células intestinais de suínos para a caracterização celular quanto a expressão de RNAm de genes relacionados á formação e desenvolvimento pancreático e ao metabolismo da insulina. O tecido intestinal e as células isoladas de leitões neonatos foram avaliados quanto a expressão de RNAm dos genes pancreáticos por qRT-PCR. O tecido e as células isoladas antes e depois do cultivo, nas duas primeiras passagens, foram avaliados para os genes Pdx1 (*Pancreatic and duodenal homeobox*), Ngn3 (*Neurogin3*), NeuroD1 (*Neurogin differentiation 1*), PC1/3 (*Prohormone convertase 1/3*), PC2 (*Prohormone convertase 2*) e o gene da insulina (INS). O intestino e a passagem 2 das células cultivadas foram avaliadas quanto a presença de insulina por imunofluorescência. No primeiro experimento, as células intestinais foram cultivadas em meio contendo RPMI, EGF, penicilina, anfotericina B e insulina (10µg/ml). Para os genes Ngn3, PC1/3 e PC2 a expressão de RNAm não diferiu durante o processo de obtenção das células nem durante as passagens, enquanto que para os genes Pdx1, INS e NeuroD1 a expressão diminuiu nas células cultivadas. No segundo experimento, foi realizado um cultivo primário de células intestinais de suínos neonatos, utilizando o meio básico descrito no primeiro experimento, mas sem insulina (grupo controle) ou com 25 mM de glicose e sem insulina (grupo tratamento). Esse experimento foi realizado com o objetivo de avaliar a expressão desses genes em resposta á ausência da insulina ou presença de glicose no cultivo. Independentemente da adição de glicose, houve uma diminuição da expressão de todos os genes estudados ao longo da primeira passagem e um aumento (retornando aos níveis iniciais encontrados no intestino), ou até superiores (NeuroD1) em células da segunda passagem, com exceção do Pdx1. A quantidade de mRNA para Pdx1 observada no tecido intestinal se manteve nas células isoladas e na primeira passagem, independente da presença de glicose, e decresceu nas células da segunda passagem. Anticorpos para insulina não detectaram a presença desta proteína no tecido intestinal, bem como nas células cultivadas com adição de glicose e do grupo controle. Com base nesses resultados, pode-se inferir que as células intestinais duodenais de suínos neonatos possuem um caráter de célula progenitora pancreática, em função do padrão de expressão gênica, porém não são capazes de produzir a proteína insulina. No entanto, observando os dados de expressão de RNAm é possível sugerir que essas células podem ser mais facilmente diferenciadas e consequentemente usadas em estudos de terapia celular para diabetes mellitus tipo 1.

Palavras-chave: Insulina. Suíno. Expressão gênica. Cultivo celular.

ABSTRACT

Master's Dissertation
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

INTESTINAL CELL POTENTIAL AS SOURCE OF PANCREATIC PROGENITOR CELLS

AUTHOR: WERNER GIEHL GLANZNER
ADVISOR: PAULO BAYARD DIAS GONÇALVES
Date and Place of Defense: Santa Maria, february 24th, 2012.

The objective of the present study was to establish a primary culture of intestinal cells and to characterize the mRNA expression profile of genes related to pancreatic formation, development and insulin metabolism. The mRNA expression of pancreatic-related genes was evaluated in intestinal tissue and isolated cells from newborn piglet by qRT-PCR. The tissue and isolated cells before and after culture from the first two passages were evaluated for Pdx1 (Pancreatic and duodenal homeobox), Ngn3 (Neurogin3), NeuroD1 (Neurogenic differentiation 1), PC1/3 (Prohormone convertase 1/3), PC2 (Prohormone convertase 2) and insulin (INS) gene expression. The intestinal tissue and passage 2 of cultured cells were evaluated for the presence of insulin by immunofluorescence. In the first experiment, cells were cultured in RPMI medium plus EGF, penicillin, amphotericin B, and insulin (10 µg/ml). The Ngn3, PC1/3 and PC2 expression did not differ during cell isolation process and culture passages, while for genes Pdx1, INS and NeuroD1 the expression decreased in cultured cells. In the second experiment, a primary cell culture of porcine newborn intestinal cells were performed using the same medium describe above but without insulin (control group) or with glucose (25 mM) and without insulin (treatment group). The objective of this experiment was to evaluate the mRNA expression of pancreatic-related genes in response to glucose. Independently of the presence of glucose, the expression of all studied genes decreased at passage 1 and raised (to the same levels found in intestine) or even higher (NeuroD1) at passage 2 of cultured cells, except for Pdx1. The Pdx1 mRNA expression observed in intestinal tissue was maintained through first cell passage, but decreased at passage 2. The intestinal tissue and cells cultured with or without glucose from the second passage did not reveal any insulin-producing cell by immunofluorescence. Our results let us to conclude that newborn duodenal tissue had cells that express mRNA pancreatic markers; however, these cells were not able to produce insulin. The results of this study allowed us to infer that newborn piglet duodenal cells have potential to be transdifferentiated in insulin producing cells for cell therapy of type 1 diabetes mellitus.

Key words: Insulin. Porcine. Gene expression. Cell culture.

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LISTA DE ABREVIATURAS

ADA: American Diabetes Association

AMPc: cyclic adenosine monophosphate

ANP: atrial natriuretic peptide

bHLH: basic helix-loop-helix transcript factor

CaMK: Ca^{2+}/calmodulin-dependent protein kinase

C/RIPE3b: C enhancer/rat insulin II enhancer

CPE: carboxipeptidase E

EGF: epidermal growth factor

GC-A: guanylate cyclase A

GFP: green fluorescent protein

GH: growth hormone

GLP: glucagon like peptide

GMPc: cyclic guanosine monophosphate

GWAS: genome wide association study

INS: gene da insulina

MafA: V-maf musculoaponeurotic fibrosarcoma oncogene homologue A

NEFA: non-esterified fatty acid

NeuroD1: Neurogenic differentiation 1

Ngn3: neurogin3

p38MAPK: P38 mitogen-activated protein kinase

PC1/3: prohormone convertase 1/3

PC2: prohormone convertase 2

PDGF: plateled-derived growth factor

Pdx1: pancreatic and duodenal homeobox

PI3K: phosphatidylinositol 3-kinase

PKA: protein kinase A

PRL: prolactin

qRT-PCR: quantitative reverse transcriptase polymerase chain reaction

SKP: skin derived progenitor

SV40: Simian virus 40

WHO: World Health Organization

SUMÁRIO

INTRODUÇÃO

A diabetes é uma desordem metabólica de impacto internacional e em crescimento na maioria dos países. Inúmeras são as consequências de problemas sistêmicos observados em pessoas e animais diabéticos. Alterações metabólicas como doenças cardíacas, renais e problemas de visão estão relacionadas com a diabetes mellitus. Estimativas de órgãos de saúde mundiais evidenciam que os gastos com a diabetes e com doenças relacionadas atinjam bilhões de dólares em todo mundo.

O tratamento mais funcional e utilizado para manter níveis de glicose dentro da faixa fisiológica é a administração de insulina nos pacientes. No entanto, transplantes de ilhotas pancreáticas associado a drogas imunossupressoras (O'sullivan *et al.*, 2011) têm sido exaustivamente estudados e também utilizados em alguns casos. Xenotransplantes, principalmente de ilhotas de suínos (Dufrane e Gianello, 2011), têm ganhado cada vez mais atenção no panorama científico com as técnicas de transgenia, as quais, permitem controlar as interações genéticas responsáveis por casos de rejeição. Apesar destes diversos tratamentos, o imenso avanço na área de células-tronco e bioengenharia de tecidos, tem permitido que cada vez mais pesquisadores estudem diferentes tipos celulares na busca de alternativas para terapia celular e estudos de diferenciação e transdiferenciação celular em busca de uma possível cura da diabetes mellitus, principalmente do tipo 1.

Desde o primeiro trabalho que obteve sucesso no isolamento de células-tronco embrionárias humanas (Thomson *et al.*, 1998), inúmeros trabalhos têm surgido utilizando células embrionárias humanas, células pluripotentes induzidas (iPSC) (Takahashi e Yamanaka, 2006) e células-tronco adultas com o intuito de gerar células capazes de produzir linhagens pancreáticas ou ainda capazes de produzir insulina *in vitro* e *in vivo*, respondendo a elevadas concentrações de glicose. No entanto, muito cuidado deve ser tomado quanto a utilização de técnicas de terapia celular, pois formação de tumores já foram descritas a partir de células fetais administradas em humanos (Amariglio *et al.*, 2009), além do potencial de formação de tumores que as células-tronco embrionárias possuem (Hentze *et al.*, 2009; Su *et al.*, 2011). O fígado e o intestino têm sido identificados como órgãos com um grande potencial de fonte de células-tronco e progenitoras, as quais expressam fatores de transcrição essenciais no desenvolvimento pancreático (Fujita *et al.*, 2004; Zaret, 2008).

Até o momento somente estudos utilizando células intestinais de animais de laboratório foram conduzidos, no entanto, nenhum estudo utilizou células intestinais de

humanos ou animais com a fisiologia mais similar aos humanos, para caracterização e transdiferenciação em relação a células progenitoras pancreáticas, apesar da proximidade quanto a origem e os fatores expressos nesses dois tipos celulares. Além disso, a possibilidade de teratoma é minimizada em relação as células pluripotentes atualmente utilizadas em investigações para a geração de células produtoras de insulina. Sendo assim, nossa hipótese é que as células intestinais de suínos expressam RNAm, isto é, possuem a maquinaria transcricional necessária para produzir fatores e proteínas relacionadas a atividade e metabolismo pancreático. Portanto, o objetivo deste trabalho foi avaliar a presença da proteína e expressão de RNAm do gene (*INS*) insulina, bem como a expressão de RNAm dos genes *Pdx1*, *Ngn3*, *NeuroD1* e das enzimas *PC1/3* e *PC2* no tecido intestinal e em cultivo primário de células intestinais.

REVISÃO BIBLIOGRÁFICA

Segundo a Organização Mundial de Saúde (Who, 2011), a diabetes mellitus é uma enfermidade que ocorre quando o pâncreas não produz insulina, ou quando o corpo não consegue utilizar a insulina produzida. A partir desta problemática existem dois tipos de diabetes: do tipo 1 quando a insulina é produzida em pequenas quantidades ou ainda não é produzida e a do tipo 2, quando a insulina não atua efetivamente.

A diabetes do tipo1 é insulina-dependente, e neste tipo especifico de diabetes, as células β pancreáticas, produtoras de insulina, são destruídas por reações auto-imunes, resultando, assim, na deficiência de insulina (Slack, 1995; Segev *et al.*, 2004; Murtaugh, 2007; Van Hoof *et al.*, 2009; Aguayo-Mazzucato e Bonner-Weir, 2010). De acordo com a Associação Americana de Diabetes (Ada, 2012), a diabetes do tipo 1 é geralmente diagnosticada em crianças e jovens adultos e é previamente conhecida como diabetes juvenil. Nos últimos anos, inúmeros estudos tem mostrado uma série de mutações e alterações genéticas associadas a diabetes do tipo 1 (Polychronakos e Li, 2011), através de estudo de associação de alterações genéticas e mutações do genoma (GWAS). A diabetes do tipo 2 resulta em deficiência relativa ou absoluta de insulina, baseada na resistência à insulina circulante. A resistência à insulina que precede a diabetes do tipo 2 é associada a anormalidades genéticas em alguns indivíduos, mas na maioria dos casos é em consequência à obesidade, em particular ao aumento de gordura visceral (Lebovitz, 1999).

A Organização Mundial de Saúde estima que o gasto com a diabetes e doenças cardíacas atinja US$ 49,2 bilhões nos próximos 10 anos (Who, 2011). Nos Estados Unidos, estatísticas atuais revelam que mais de 23 milhões de pessoas (8% da população) são portadoras da diabetes, com um crescimento de 14% no período entre 2005 e 2007 (Ada, 2011). Ainda, segundo dados da ADA, as maiores complicações relacionadas a essa enfermidade são: doenças cardíacas, hipertensão arterial, cegueira, falência renal, neuropatias e casos de amputações de extremidades devido a problemas circulatórios periféricos.

Desenvolvimento Pancreático

O pâncreas é um órgão que apresenta uma grande importância para o status nutricional dos humanos e animais, porque sintetiza e secreta enzimas e hormônios que atuam nessa função (Collombat *et al.*, 2006). As enzimas que atuam no auxílio a digestão são sintetizadas pelo pâncreas exócrino, que compreende os ácinos pancreáticos, os quais secretam enzimas, como lipases, amilases, tripsinogênio e quimotripsinogênio, na luz intestinal. Os hormônios insulina, glucagon, somatostatina e polipeptídio pancreático são produzidos pelo pâncreas endócrino e seus produtos, secretados na corrente circulatória (Slack, 1995). O tecido endócrino é organizado em um grupo de células denominadas *Ilhotas de Langerhans* (Habener *et al.*, 2005; Collombat *et al.*, 2006). Cada ilhota de Langerhans possui quatro tipos celulares, α, β, δ e as células PP, que produzem e secretam glucagon, insulina, somatostatina e o polipeptídio pancreático, respectivamente (Collombat *et al.*, 2006).

Para a formação do pâncreas ocorrem basicamente duas invaginações, uma dorsal e outra ventral, a partir do intestino delgado (duodeno) que em camundongos, foi observado, por volta dos 9,5 dias de vida embrionária (Habener *et al.*, 2005; Collombat *et al.*, 2006; Gittes, 2009). Em humanos, essas invaginações para inicio da formação do pâncreas foram observados ao redor dos 26 dias de gestação (Gittes, 2009), ou seja, entre a 3ª e a 4ª semana (Van Hoof *et al.*, 2009). Para a formação do pâncreas como um único órgão, as duas invaginações, dorsal e ventral, devem se unir através da rotação do estômago e do duodeno (Slack, 1995), o que em camundongos acontece por volta dos 12-13 dias e, em humanos, por volta dos 42 dias do desenvolvimento embrionário (Gittes, 2009; Van Hoof *et al.*, 2009). Uma abordagem para o estudo de expressão de insulina foi realizado através da produção de camundongos transgênicos capazes de expressar o gene GFP pela estimulação do promotor do gene da insulina, permitindo identificar os períodos de desenvolvimento embrionário e fetal em que o gene da insulina é expresso (Hara *et al.*, 2003).

Durante o desenvolvimento fetal, inúmeros genes expressos coordenam os passos da diferenciação do pâncreas e formação das células pancreáticas e endócrinas (Bernardo *et al.*, 2008; Oliver-Krasinski e Stoffers, 2008; Zaret, 2008; Gittes, 2009). Dentre os genes relacionados ao desenvolvimento e diferenciação pancreática, destacam-se o *Pdx1* e *Ngn3*. O gene *Pdx1* é um dos principais genes expressos durante a formação do órgão pancreático, uma vez que animais *knockout* para esse gene apresentam ausência do órgão (Jonsson *et al.*, 1994; Offield *et al.*, 1996). Esses animais apresentaram retardo de crescimento ao primeiro dia de

vida e uma forte desidratação, além de malformações no duodeno e estômago e dificuldade no esvaziamento gástrico (Offield *et al.*, 1996). Em camundongos, foi observado que células do vilo intestinal, mas não da cripta, expressam *Pdx1* (Guz *et al.*, 1995), evidenciando que a expressão desse gene se evidencia conforme as células se diferenciam e migram das criptas para o vilo. A expressão de *Pdx*1 pode ser vista a partir dos 8,5 dias fetais em camundongos (Guz *et al.*, 1995) e aos 19 dias fetais em suínos (Carlsson *et al.*, 2010) nos tecidos que darão origem ao pâncreas. Chen et al. (2009) utilizando *knockout* condicional restrito ao epitélio intestinal, observaram alterações na expressão de genes de células enteroendócrinas no intestino proximal, porém sem maiores alterações de órgãos desta região. Em humanos, uma deleção de base única encontrada em homozigose no gene do *Pdx1* é responsável por agenesia pancreática; no entanto, em heterozigose, o fenótipo do individuo portador da mutação não é afetado (Stoffers *et al.*, 1997). Uma das funções do gene *Pdx1* seria contribuir para especificação dos progenitores endócrinos regulando o gene *Ngn3* diretamente, além de participar de uma rede de multi-regulação de fatores de transcrição durante o desenvolvimento precoce do pâncreas (Oliver-Krasinski *et al.*, 2009). O gene *Ngn3* é um dos genes principais no desenvolvimento pancreático, sendo considerado um gene marcador de células progenitoras endócrinas do pâncreas (Gradwohl *et al.*, 2000; Schwitzgebel *et al.*, 2000; Gu *et al.*, 2002). Além de progenitor endócrino pancreático, o *Ngn3* seria marcador de células progenitores endócrinas da região proximal do intestino, com uma expressão restrita a cripta intestinal em animais adultos (Jenny *et al.*, 2002). Animais *knouckout* para esse gene não apresentaram formação de células endócrinas pancreáticas (Gradwohl *et al.*, 2000). Em camundongos, a expressão de Ngn3 foi observado em embriões a partir dos 11,5 dias de desenvolvimento, atingindo uma maior proporção aos 15,5 e uma diminuição a partir dos 18,5 dias de desenvolvimento fetal, não sendo expresso em pâncreas de animais adultos (Schwitzgebel *et al.*, 2000). O *Ngn3* é regulado pelo sistema de sinalização Notch (Apelqvist *et al.*, 1999), sistema que esta relacionado à diferenciação, proliferação e apoptose celular de diversos sistemas do organismo animal (Artavanis-Tsakonas *et al.*, 1999).

Assim como o *Pdx1* e o *Ngn3*, outros genes são essenciais para a regulação da transcrição e consequente secreção de insulina. Os genes *Pdx1*, juntamente com o *NeuroD1* e o *MafA*, são fatores que atuam aumentando a transcrição do gene da insulina em resposta a elevação das concentrações de glicose (Andrali *et al.*, 2008). Essas funções ocorrem devido a interações com histonas deacetilases e ligações especificas nas regiões regulatórias do promotor do gene da insulina. O fator de transcrição *NeuroD1* também descrito como *BETA2* parece ser regulado basicamente pelo *Ngn3* (Huang *et al.*, 2000). Animais *knouckout* em

homozigose para esse fator de transcrição morrem após 5 dias do nascimento, apresentam diabetes severa não capaz de responder ao tratamento com insulina exógena, possivelmente em função de uma resistência à insulina ou outras alterações e apresentam formação anormal das ilhotas e células endócrinas, evidenciando a participação deste gene nas linhagens endócrinas (Naya *et al.*, 1997). Portanto, há evidências de que o *NeuroD1* compartilhe juntamente com o *Ngn3* um papel, na formação de células de linhagens endócrinas (Gasa *et al.*, 2004). Drogas que induzem a expressão de *NeuroD1* estão relacionadas com o aumento da função das células β aumentando, desta forma, a produção e consequente liberação de insulina (Dioum *et al.*, 2011).

Insulina

A insulina foi descoberta em 1922 por um médico Canadense chamado Frederick Banting e seu colaborador Charles Best (Banting e Best, 1922), trabalhando com extrato pancreático perceberam que esse era capaz de inibir os efeitos da diabetes em cães. Uma série de estudos se seguiu (Banting *et al.*, 1922; Banting *et al.*, 1923), e em 1923 Frederick Banting recebeu o Premio Nobel em Fisiologia.

O gene da insulina transcreve para uma pré-proteína denominada preproinsulina. A preproinsulina é composta por um peptídeo sinal, duas cadeias (B e A), que formarão a insulina madura, as quais são ligadas pelo peptídeo conectante (peptídeo C) (Steiner *et al.*, 1985; Yang *et al.*, 2010b). À medida que a preproinsulina vai sendo traduzida, a clivagem do peptídeo sinal vai sendo realizada, formando a proinsulina (Steiner *et al.*, 1985; Steiner *et al.*, 2009). A proinsulina é, então, armazenada no complexo de Golgi (Steiner *et al.*, 1985; Steiner *et al.*, 2009), onde é clivada pelas enzimas *PC1/3*, (*PC2*) e carboxipeptidade E (*CPE*) (Steiner, 2004; Steiner *et al.*, 2009). Após a clivagem da proinsulina, é liberado o peptídeo C e a insulina madura, em forma de vesículas, com as cadeias A e B que possuem 21 e 30 aminoácidos respectivamente, com algumas variações de aminoácidos em algumas espécies (Kaneko, 2008).

O peptídeo C, liberado na clivagem da proinsulina, é secretado em quantidades equimolares à insulina na circulação, uma vez que sua liberação resulta na formação da insulina madura (cadeias A e B) (Steiner, 2004). O papel do Peptídeo C é ainda controverso, alguns pesquisadores defendem uma função como peptídeo ativo (Wahren *et al.*, 2007), atuando a fim de minimizar danos renais e nervosos causados pela diabetes do tipo 1, através

da ativação de mensageiros secundários e fatores de transcrição, porém sem uma função clara e conhecida em indivíduos normais, sem deficiência de insulina.

O gene da insulina em humanos possui três exons e dois íntrons, sendo o tamanho em pares de base da região dos íntrons extremamente variável entre diferentes espécies. Ainda que na maioria das espécies exista somente um gene da insulina com a descrição acima, ratos e camundongos possuem dois genes para insulina, nos cromossomos 1 em ratos e 6 e 7 em camundongos (Steiner *et al.*, 1985). Inúmeros nutrientes e hormônios parecem atuar aumentando ou diminuindo a expressão e liberação da insulina como a glicose, NEFA, GLP-1, insulina, leptina, GH e PRL. Esses nutrientes e hormônios atuam basicamente através da ativação de mensageiros secundários dentro das células como: PKA, PI3K, AMPc, p38MAPK e CaMK, que por sua vez se ligam a regiões reguladoras ou ativam fatores de transcrição a se ligarem nessas regiões regulando a expressão da insulina (Melloul *et al.*, 2002). A regulação da expressão do gene da insulina é controlada através da interação de sequências regulatórias do promotor do gene ou próximo ao promotor com fatores de transcrição específicos das células beta (Melloul *et al.*, 2002; Andrali *et al.*, 2008). Segundo Andrali et al. (2008), três fatores de transcrição que atuam de maneira efetiva aumentando a transcrição do gene da insulina em função do aumentos dos níveis de glicose são o *Pdx1, NeuroD1* e o *MafA*, os quais atuam nas regiões reguladoras A3, E1 e C1, respectivamente e aumentam a transcrição da preproinsulina quando os níveis de glicose estão aumentados. Embora o gene da insulina possua várias regiões reguladoras, as regiões A, E e C/RIPE3b (rat insulin II enhancer), esta última composta por dois elementos regulatórios distintos: A2 e C1 (Melloul *et al.*, 2002), são mais importantes e, portanto, mais estudadas. A região regulatória E parece ser um elemento regulador responsável pela ligação dos fatores de transcrição da família basic helix-loop-helix (bHLH) (Naya *et al.*, 1995).

Recentemente, foi observado que nas células β, o peptídeo natriurético A (ANP) é capaz de induzir a secreção de insulina e que este evento pode ter uma ação sinérgica com a estimulação pela glicose (Ropero *et al.*, 2010). Isso ocorre em consequência da ativação da GC-A pelo ANP, aumentando os níveis de GMPc que induziria o fechamento dos canais de potássio dependentes de ATP (K_{ATP}). O fechamento dos canais de potássio seria responsável pelo aumento das concentrações de cálcio intracelular. Os mecanismos envolvidos na liberação da insulina madura, pela célula beta, induzida pela glicose envolvem o fechamento dos canais K_{ATP} em função do aumento da relação ATP/ADP dentro célula, o que causa uma despolarização da membrana aumentando a permeabilidade dos canais de cálcio. Consequentemente, o cálcio intracelular, resultaria em exocitose das vesículas contendo

insulina (Soria *et al.*, 2004). A glicose além de atuar na liberação de insulina parece atuar no aumento da transcrição (Nielsen *et al.*, 1985) e na estabilidade do RNA para esse gene através da ação do AMPc (Welsh *et al.*, 1985).

Tratamento da Diabetes e terapia celular

Na tentativa de entender melhor e controlar a diabetes, principalmente a diabetes mellitus do tipo 1, três abordagens têm sido adotadas 1) estudos básicos para o entendimento e controle do sistema auto-imune (Kent *et al.*, 2005; Liu *et al.*, 2009; Pechhold *et al.*, 2009), 2) produção de células β para transplantes (Sordi *et al.*, 2008; Zhou *et al.*, 2008; Li *et al.*, 2009) e 3) estímulo a regeneração de células β pancreáticas (Bonner-Weir e Weir, 2005; Nir *et al.*, 2007; Bonal *et al.*, 2008). Nesse sentido, os estudos sobre a ontogenia de células pancreáticas demonstraram a importância de alguns fatores de transcrição (Mellitzer *et al.*, 2006; Bernardo *et al.*, 2008) e fatores de crescimento (Bhushan *et al.*, 2001; Elghazi *et al.*, 2002; Ye *et al.*, 2005; Calderari *et al.*, 2007) sobre o desenvolvimento do pâncreas. Apesar dos progressos obtidos no transplante de ilhotas após os primeiros estudos em roedores (Ballinger e Lacy, 1972) e do sucesso do protocolo de Edmonton em humanos (Shapiro *et al.*, 2000), os principais obstáculos ainda não foram ultrapassados. O índice de receptores que alcançam a independência de insulina com adequado controle da glicemia é de apenas 44% no primeiro ano (Cernea e Pozzilli, 2008), 31% no segundo ano (Lu *et al.*, 2007) e 10% no quinto ano (Ryan *et al.*, 2005). As principais razões de perdas em ilhotas transplantadas estão relacionadas com os procedimentos de isolamento das ilhotas, toxicidade dos imunosupressores, reações inflamatórias e rejeições (Titus *et al.*, 2000; Shapiro *et al.*, 2003). Sendo os problemas de rejeição o maior obstáculo para difusão dos transplantes de ilhotas (Robertson, 2004; Jiang *et al.*, 2008). Na área de transplante de ilhotas pancreáticas o que tem crescido são os trabalhos relatando xenotransplantes, principalmente de suínos (Dufrane e Gianello, 2008; Dufrane e Gianello, 2011) que possuem morfofisiologia muito semelhante aos humanos, podendo esta ser uma terapia possível no futuro a partir de criação de animais nos sistemas *germ free.* Estudos recentes mostram que algumas drogas e fatores de crescimento podem vir a ser uma alternativa no tratamento da diabetes, porem com maior foco a diabetes mellitus tipo 2, como o PDGF (Chen *et al.*, 2011), agonistas do peptídeo semelhante ao glucagon como o caso da liraglutida (Kela *et al.*, 2011; Ryan e Hardy, 2011), ou ainda a família das isoxazoles (Dioum *et al.*, 2011), embora tais drogas e fatores de crescimento

possam vir a ser usados também em estudos de diferenciação celular *in vitro* visando terapia celular para diabetes mellitus do tipo 1.

Ultimamente, uma abordagem que vem sendo utilizada na busca de possíveis terapias para a diabetes é a terapia celular, baseada na diferenciação de células embrionárias em células pancreáticas capazes de produzir insulina. Essas células previamente diferenciadas, são, então, injetadas nos animais promovendo o aumento dos níveis de insulina e estabilização da glicose (Jiang *et al.*, 2007b; Shim *et al.*, 2007; Kroon *et al.*, 2008). Uma das maiores desvantagens na utilização de células-tronco embrionárias é o alto potencial de formação de teratomas e tumores (Kahan *et al.*, 2011; Su *et al.*, 2011), e mesmo células diferenciadas previamente *in vitro* têm demonstrado esse potencial (Kroon *et al.*, 2008), principalmente devido a anormalidades cromossômicas e reprogramação a que essas células estão sujeitas (Aguayo-Mazzucato e Bonner-Weir, 2010) em função dos sistemas de cultivo. Com isso, busca-se nas células-tronco adultas e/ou progenitoras uma possível tentativa de diferenciação em células com caráter pancreático, ou seja, que produzam insulina para repovoamento do pâncreas a partir de células diferenciadas e competentes, mas com menor potencial oncogênico e uma consequente redução no número de formações tumorais a partir dessa abordagem.

Estudos de diferenciação e transdiferenciação celular

A diferenciação celular normalmente é um processo irreversível, com algumas exceções encontradas na natureza, denominado como transdiferenciação, desdiferenciação ou transdeterminação, que ocorrem nos vertebrados com maior capacidade de regeneração (ex. Anfíbios) (Hadorn, 1968; Brockes e Kumar, 2002; Odelberg, 2002; Tanaka, 2003), em invertebrados (ex. Drosophila) (Sustar e Schubiger, 2005; Mcclure *et al.*, 2008) e em alguns casos de metaplasias (Slack, 2007).

Inúmeros trabalhos têm surgido desenvolvendo protocolos para diferenciação de células-tronco embrionárias em células pancreáticas, ou capazes de secretar insulina (D'amour *et al.*, 2006; Jiang *et al.*, 2007a; Jiang *et al.*, 2007b; Phillips *et al.*, 2007; Shim *et al.*, 2007; Kroon *et al.*, 2008; Zhang *et al.*, 2009), sendo já realizado a diferenciação de células embrionárias derivadas de produtos clonados em células pancreáticas também capazes de produzir insulina (Jiang *et al.*, 2008), ou ainda a diferenciação de células pluripotentes induzidas (iPSC) em produtoras de insulina (Zhang *et al.*, 2009).

No entanto, apesar de ser possível a diferenciação de células embrionárias, iPSC, ou células reprogramadas por clonagem, o potencial tumoral ainda é um obstáculo. Com isso, o processo de transdiferenciação tem sido recentemente observado e induzido em mamíferos, estando entre as novas promessas para a substituição de células β (Odelberg, 2002; Baeyens *et al.*, 2005; Bonner-Weir e Weir, 2005; Heimberg, 2008; Zhou *et al.*, 2008; Motoyama *et al.*, 2009). Os órgãos derivados do endoderma têm sido relacionados entre aqueles com grande potencial para servir como fonte de células capazes de se diferenciar em células produtoras de insulina para o tratamento da diabetes. Essas células normalmente expressam fatores de transcrição essenciais no desenvolvimento e manutenção da função pancreática (Fujita *et al.*, 2004; Zaret e Grompe, 2008).

Dentre esses órgãos, destacam-se estudos em humanos onde pesquisadores obtiveram células produtoras de insulina em linhagens imortalizadas através de vetores virais com o vírus SV40 e RT-telomerase a partir de células de fígado fetal (Zalzman *et al.*, 2003), sendo que essas mesmas células foram capazes a responder a estímulos de glicose *in vivo* (Zalzman *et al.*, 2005). Ainda em linhagens de órgãos do endoderma, um trabalho utilizando a mesma metodologia de vetores virais para imortalização de linhagens celulares de pâncreas humano que responderam a desafios de glicose (25mM) *in vitro* e *in vivo* (Narushima *et al.*, 2005). Células pancreáticas exócrinas foram reprogramadas em células endócrinas pancreáticas capazes de produzir insulina (Zhou *et al.*, 2008), e ainda a diferenciação de células-tronco mesênquimais da medula de ratos em células β-pancreáticas (Chen *et al.*, 2004; Oh *et al.*, 2004; Neshati *et al.*, 2010; Yuan *et al.*, 2010) já foram realizadas.

Em relação a células intestinais, Suzuki et al. (Suzuki *et al.*, 2003), trabalhando com intestinos de camundongos e utilizando peptídeo tipo glucagon (GLP), produziram células produtoras de insulina e responsivas à glicose a partir de células intestinais, porém não observaram expressão de insulina no intestino desses animais em condições fisiológicas. Em suínos, poucos são os trabalhos disponíveis até o momento sobre diferenciação celular e terapia celular, sendo que células progenitoras da pele (SKP) de suínos criados em sistema livre de patógenos (Yang *et al.*, 2010a) já foram diferenciadas em células produtoras de insulina. Em suínos outro trabalho relata que células do fígado foram capazes de expressar genes relacionados ao desenvolvimento do pâncreas, e responderam a desafio de glicose com o auxilio de Exendin4, um agonista do GLP (Racanicchi *et al.*, 2007).

O suínos representam um dos melhores modelos animais para estudos de doenças de humanos, devido a similaridades fisiológicas. Além disso, pouca literatura é hoje disponível visando protocolos de diferenciação celular e terapia celular com foco na diabetes, nesta

espécie. Sendo assim nosso grupo levantou a hipótese de que células do tecido intestinal de suínos em cultivo expressam genes relacionados à formação, desenvolvimento e metabolismo pancreático, e desta forma, podem ter potencial de produzir insulina, *in vitro* e responder a desafios de glicose. Para isso, o objetivo do presente estudo foi caracterizar a expressão de RNAm de genes essenciais para o desenvolvimento e formação pancreática como o *Pdx1*, diferenciação endócrina das células pancreáticas como o *Ngn3* e *NeuroD1*, metabolismo da insulina com os genes *PC1/3* e *PC2* e o gene da insulina no tecido intestinal de suínos neonatos, na suspensão de células utilizadas em um cultivo primário de células intestinais e nas duas primeiras passagens do cultivo celular, e ainda avaliar a presença da insulina no intestino e nas passagens do cultivo.

ARTIGO CIENTÍFICO

TRABALHO A SER ENVIADO PARA PUBLICAÇÃO:

INTESTINAL CELLS AS A SOURCE OF PANCREATIC PROGENITORS CELLS

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Intestinal cells as a source of pancreatic progenitor cells

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Running Head: Intestinal cells as pancreatic progenitor cells

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Abstract

The aim of this study was to verify the mRNA expression of pancreatic-related genes in intestinal tissue and cultured cells from newborn porcine duodenal tissue. Intestinal segments were used either for RNA extraction or for a primary cell culture. Cells were cultured in the presence of insulin in the first experiment and without insulin and either without glucose (control group) or with 25 mM of glucose (treatment group) in the second experiment. The isolated duodenal cells were analyzed by qRT-PCR and the presence of insulin was evaluated by immunofluorescence. Cells cultured in the presence of insulin

revealed downregulation in *INS*, *Pdx1* and *NeuroD1* mRNA expression; however, the expression of *Ngn3*, *PC1/3* and *PC2* mRNA did not differ in insulin-cultured cells. In the second experiment, we observed a downregulation in the mRNA expression of all studied pancreatic markers, with the exception of *Pdx1*, during the first cell passage, but an upregulation during the second passage. Interestingly, the expressions of *Pdx1* mRNA in isolated intestinal cells after the first passage did not differ from those observed in intestinal tissue, decreasing at passage 2. The intestinal tissue and cells cultured with or without glucose from the second passage did not reveal any insulin-producing cell by immunofluorescence. Our results allow us to infer that newborn piglet duodenal cells have molecular machinery to be a source of insulin producing cell.

Key words: Insulin; Porcine; Gene expression; Cell culture.

Introduction

The progressive loss of β cells in Type 1 Diabetes (T1D) is caused by a chronic autoimmune attack. Despite advances in treatment of diabetes and blood glucose control, secondary injuries affecting eyes, kidneys, nerves and cardiovascular diseases are frequently associated with diabetes. Insulin therapy seems to be insufficient to avoid these long-term consequences. In an attempt to better understand and control T1D, three distinct approaches have been adopted with well-designed experiments based on animal models: a) understand and control the autoimmune system (1-3), b) generation of β-cells ex vivo for transplantation (4-6) and c) stimulate pancreatic β-cells regeneration (7-9). Even though great progress in the islet transplantation has already been achieved after the first studies in rodents (10) and considering the success of the Edmonton Protocol in humans (11), the main obstacle were not yet overcome. In turn, the actual rate of recipients that achieve insulin independence with adequate glycemic control is only 44% in the first year (12), decreasing to 31% in the second

52 year (13) and 10% in the fifth year (14) after transplantation. The major reasons for loss of
53 transplanted islets are isolation procedures, immunosuppression toxicity, inflammatory
54 reaction and rejection (15, 16). Nevertheless, the supply of islets is insufficient for the
55 demand and multiple donor organs per recipient are needed. These facts highlight the reasons
56 to search for new sources of β-cells for improvement of human health (17).

57 Successfully studies have demonstrated that embryonic stem cells may be
58 differentiated into insulin-producing cells (18-24). However, the tumorigenic potential of
59 fetal-derived cells (25) and embryonic stem cells (26, 27) must be carefully considered as a
60 risk in a cell therapy procedure. For this reason, other sources of cells that exhibit gene
61 expression profile compatible with differentiation in β-cells with a low risk of tumor
62 formation should be pursued. Cells from endoderm-derived organs as liver and intestine, with
63 transcriptional profiles similar to pancreatic progenitor cells, are candidates for differentiation
64 in to insulin-producing cells (28, 29); however, for porcine intestinal cells this potential is still
65 unknown. The cell differentiation is typically an irreversible process, with some exceptions
66 found in nature, termed as transdifferentiation, dedifferentiation or transdetermination, which
67 occur in vertebrates with greater capacity for regeneration (e.g. Amphibians) (30-33), in
68 invertebrates (e.g. Drosophila) (34, 35) and in some cases of metaplasia (36). This way, the
69 process of transdifferentiation has recently been observed or induced and considered as a new
70 promise for β-cell replacement (5, 7, 37-39).

71 Studies on the pancreatic cell ontogeny have been performed and some transcription
72 factors (40) and growth factors (41-45) have been clearly demonstrated as essential for the
73 pancreatic development. From these experiments, specific markers of the primary (pancreatic
74 bud emergence, expansion and fusion) and secondary (specification of endocrine and exocrine
75 lineage and differentiation of β-cells) transitions have been determined (40). The pancreatic
76 and duodenal homeobox (*Pdx1*) is considered one of the most important markers for pancreas

77 formation and function. The *Pdx1*-null mice (46-48) and humans with homozygous mutations
78 on ortholog of the *Pdx1* gene (*Ipf1* gene) (49, 50) have pancreas agenesis. Regarding the
79 mature β cell function, mutations in the gene encoding *Pdx1* impair insulin secretion,
80 inducing the maturity onset diabetes of the young 4 (MODY4) in humans (51-55) and β cell
81 malfunction in animal models (56-58).

82 The gene neurogin3 (*Ngn3*) is considered as an endocrine progenitor cell marker (59-
83 61). Knockout animals for *Ngn3* transcript factor have no pancreatic endocrine cells (59).
84 Another transcript factor related to endocrine cells fate is the neurogenic differentiation 1
85 (NeuroD1), which is mainly regulated by *Ngn3* (62). Drugs that induce *NeuroD1* expression
86 are related to ameliorate β cell function and increase insulin production (63). *Pdx1* and
87 *NeuroD1* are known as stimulators of insulin gene transcript at high glucose concentrations
88 (64). In addition to these transcription factors, the enzymes that cleave the insulin gene
89 product are important markers for insulin-producing cells. The insulin gene transcribes to
90 preproinsulin, which is a signal peptide composed by two chains (A and B) and a connecting
91 peptide (C peptide) (65, 66). The preproinsulin is cleaved in the proinsulin, which is further
92 cleaved in mature insulin by the action of prohormone convertases 1/3 (*PC1/3*) and
93 prohormone convertase 2 (*PC2*) (67, 68).

94 The aim of the present study was to characterize swine duodenal tissue and isolated
95 duodenal cells in culture as regard to gene expression related to pancreatic development and
96 β-cell function, since swine represents an important animal model for human cell therapy
97 studies. Expression of mRNA was evaluated for the insulin gene (*INS*), *Pdx1, Ngn3,*
98 *NeuroD1, PC1/3* and *PC2* in tissue derived from the first portion of the piglet intestine, in
99 intestinal cell suspension and in intestinal cells after the first and second passages in a cell
100 culture system *in vitro*.

101

102 **Materials and Methods**

103 Intestinal tissue was obtained from piglets immediately after birth, in accordance with
104 procedures approved by the Ethics and Animal Welfare Committee of the Federal University
105 of Santa Maria (23081.006726/2011-60 CCR/UFSM). All chemicals used were purchased
106 from Sigma Chemicals Company, St. Louis, MO, USA, unless otherwise indicated in the text.
107

108 *Sample collections and cell culture*

109 Porcine duodenal tissue obtained from newborn piglets was used for RNA extraction
110 and for isolating cells to *in vitro* culture. The duodenal tissues used for the cell culture were
111 washed twice in PBS with 100 UI of penicillin and 100 µg/ml of streptomycin and cut in 3 cm
112 pieces. The serosa layer was removed to avoid any possible cross contamination with
113 pancreatic cells. The small fragments were washed in PBS and digested in a collagenase
114 solution (1 mg/ml) at an atmosphere of 5% CO_2 in air, and 37 ^{o}C for 20 min. After the
115 digestion period, the tissue fragments were washed three times in PBS and the duodenal
116 mucosa was scraped with a cell scraper (Corning®) to obtain single cells in suspension. The
117 cell suspension was centrifuged five times at 250 g in a 2% sorbitol solution for 3 min at 10
118 °C to eliminate dead cells and cellular residues (69). The cells were washed and centrifuged at
119 250 g twice in RPMI medium and resuspended in the culture medium: RPMI plus 10% fetal
120 bovine serum (FBS), 100 UI/ml of penicillin, 100 µg/ml of streptomycin, 0.25 µg/ml of
121 amphotericin B, 10 µg/ml of insulin and 20 ng/ml of epidermal growth factor (EGF). The
122 cells were seeded in 60 mm polystyrene cell culture plates (Corning®) at 2-3x10^5 viable cells
123 per plate. The cells were counted in Neubauer chamber after colored with 0.4% tripan blue. In
124 all experiments, the cells were cultured at 37 °C in an atmosphere of 5% CO_2 with saturated
125 humidity and the medium was changed every 72 hours. The cultures were monitored daily
126 with a Leica DMI 4000B inverted microscope, and when the culture was confluent the cells

127 were trypsinized with 0.25% trypsin solution. The suspended cells were centrifuged at 250 g
128 for 5 min and, separated in two aliquots. One aliquot was used for RNA extraction and the
129 other was seeded into a new culture plate.
130
131 *Cell Glucose Response*
132 The intestinal cells were cultured in presence (25 mM) or absence of glucose, without
133 insulin in order to determine the effect of glucose on cell mRNA expression during the first
134 and second culture passages. The cell culture was conducted as described above; however,
135 insulin was not added to the culture medium. The medium was changed every 72 hours and
136 the cells in each passage were collected for RNA extraction and for immunofluorescence
137 assay.
138
139 *RNA extraction and qRT-PCR*
140 Total RNA was extracted from duodenal tissue and from isolated cells before and after
141 culture from the first and second passages using Trizol reagent (Invitrogen). Total RNA was
142 quantified by absorbance at 260 nm and RNA integrity was verified electrophoretically by
143 ethidium bromide staining. Total RNA was treated with DNase (Invitrogen) at 37 °C for 5
144 min to digest any contaminating DNA. The reverse transcriptase reaction was performed with
145 1 µM oligo-dT primer, 4 U omniscript RTase (Omniscript RT Kit, Qiagen), 0.5 mM dNTP's
146 mix (Invitrogen), 10 U RNase inhibitor (Invitrogen) in a final volume of 20 µl. The relative
147 gene expression was performed by qRT-PCR using the StepOnePlus™ RT-PCR system
148 (Applied Biosystems) with Power SYBR Green PCR Master Mix (Applied Biosystems) and
149 variability in the amount of mRNA was corrected by amplification of *B2M* (Beta-2-
150 microglobulin) housekeeping gene. The calculation of relative expression was performed as
151 recommended by *Pfaffl et al.* (70). The primers for *preproINS, Pdx1, Ngn3, NeuroD1, PC1/3,*

152 *PC2* and for the housekeeping gene *B2M* (Table 1) were designed using the Primer Express
153 program v 3.3 (Applied Biosystems) based on GenBank or Ensembl sequences and
154 synthesized by Invitrogen. The Primer for *Ngn3* was designed based on maximum overlap
155 between human (NM_020999.3), bovine (XM_002698866.1) and mouse (NM_009719.6)
156 mRNA sequences.
157
158 *Immunofluorescence and cell morphology*
159 Pancreas from newborn and adult swine as well as duodenum from newborn piglet
160 were fixed in a 4% paraformaldehyde solution at 4 °C for 12 h and paraffin embedded for
161 further detection of insulin protein. Histological sections with 5 μm in thickness and slides
162 were prepared to perform immunofluorescence analysis. The cultured cells were trypsinized
163 and fixed on slides with methanol. For both tissue slides and fixed cells, a blocking solution
164 (PBS 1X with 3.0% of Bovine Serum Albumin and 0.2% Tween-20) was used to block non-
165 specific sites during 2 h at room temperature in a humidified chamber. The same blocking
166 solution was used to incubate the primary porcine insulin antibody (dilution 1:100; Santa
167 Cruz Biotechnology; sc-57339) in humidified chamber overnight at 5 °C. After the incubation
168 with primary antibody, tissues and cells were incubated with goat anti-mouse IgG antibody
169 conjugated with AlexaFluor 488 (dilution 1:400; Invitrogen) for 90 min at room temperature
170 and washed three times with PBS. Finally, samples were incubated with 300 nM of 4',6-
171 diamidino-2-phenylindole (DAPI; Invitrogen) to enable nuclear staining visualization. Then,
172 slides were mounted with a space between the coverslip, filled with 10 μl drop of Aqueous
173 Mounting Medium (Fluoromount) and sealed with nail polish. Fluorescence analysis was
174 performed on an Olympus Espectral FV1000 confocal microscope using the image software
175 FV-Viewer (Olympus).

176 Cultured cells were visualized with a Leica Microscope DMI 4000B, equipped with
177 Integrated Modulation Contrast (IMC; Leica Microsystems) and images of cultured cells were
178 obtained using Leica Application Software.
179
180 *Statistical analysis*
181 The regulation of gene expression in samples was analyzed by ANOVA and means
182 compared by least square means (LSMEANS). Data were tested for normal distribution using
183 the Shapiro–Wilk test and normalized as necessary. All analyses were performed using JMP
184 software (SAS Institute Inc., Cary, NC) and $P < 0.05$ was considered statistically significant.
185 Data are presented as means ± SEM.
186
187 **Results**
188 *mRNA expression of pancreatic-related genes in cells cultured with insulin*
189 We observed mRNA expression of the genes INS, Ngn3, Pdx1, NeuroD1, PC1/3 and
190 PC2 in duodenal tissue, isolated cell suspension and cell cultured (until the second passage) in
191 the presence of 10 μg/ml of insulin (Fig. 1). The cells isolated for culture had preserved the
192 capacity to express all the pancreatic genes evaluated. The presence of insulin in the culture
193 system affected negatively the mRNA expression of INS, Pdx1 and NeuroD1 (Fig. 1A, C, D)
194 but did not affect the Ngn3, PC1/3 and PC2 mRNA expression (Fig. 1B, E, F).
195
196 *mRNA expression of pancreatic-related genes in cells cultured in the presence of glucose*
197 In this experiment, we hypothesized that the addition of glucose in the culture medium
198 of the intestinal cell culture would improve the mRNA expression of genes required for
199 pancreatic development and function in intestinal-derived cells. Piglet duodenal cells were
200 isolated and cultured in the presence (25 mM) or absence of glucose. The isolated cells had

201 preserved their capacity to express INS, Ngn3, Pdx1 and NeuroD1 mRNA before culture but
202 the PC1/3 and PC2 mRNA accumulation was markedly reduced at this point compared to the
203 intestinal tissue (Fig. 2). Curiously, the absence of insulin in the cell culture system was more
204 marked than the presence of glucose. In cultured cells, independently of the presence or
205 absence of glucose in the culture medium, it was observed downregulation in the first and
206 upregulation in the second cell-culture passage of all pancreatic genes, with the exception of
207 Pdx1 (Fig. 2).
208
209 *Immunofluorescence and cell morphology*
210 We looked at the presence of insulin by immunofluorescence in porcine duodenal
211 tissue (from newborn and adult pig) and in isolated cell cultured from the second passage, for
212 the reason that insulin is a product from multiple post-translational cleavages and the mRNAs
213 of enzymes PC1/3 and PC2 were present in intestinal cells and were upregulated in cultured
214 cells, (Fig. 3). Insulin was observed in the cell cytoplasm of adult and newborn pancreas
215 (positive control; Fig. 3A-F). However, the protein was detected neither in newborn
216 duodenum (Fig. 3G-I) nor in duodenal cultured cells (data not showed). The cell morphology
217 was normal during cell culture passages and between treatments. Cells that were cultured in
218 the absence of insulin and glucose (Fig. 3J) showed similar fibroblastic shape morphology as
219 cells treated in the presence of 25 mM of glucose (Fig. 3K).
220
221 **Discussion**
222 Our significant findings are: (a) several genes that are required for pancreatic
223 development and function (*INS, Ngn3, Pdx1, NeuroD1, PC1/3 and PC2*) were expressed by
224 piglet intestinal tissue; (b) intestinal-derived cells isolated for culture preserved the capacity
225 of expressing these pancreatic-related genes; (c) glucose did not improve the mRNA

226 expression of pancreatic-related genes by intestinal cells *in vitro* and (d) insulin was not
227 present in piglet duodenal tissue or isolated cultured cells.

228 The first experiment was conducted to characterize the profile of pancreatic-related
229 genes mRNA expression in intestinal cells cultured in the presence of insulin in relation to
230 intestinal tissue before culture. *In vivo*, other studies demonstrated that exogenous insulin or
231 increased insulin levels induce a decrease of insulin secretion or *INS* mRNA expression (71-
232 74). In agreement, we observed that the expression of *INS,* and other genes associated with
233 insulin production and secretion (*Pdx1* and *NeuroD1*) in intestinal cultured cells decreased
234 when insulin was present in the cell culture system. On the other hand, the mRNA expression
235 of *Ngn3, PC1/3* and *PC2* were present at a constant level during the culture period for cells
236 cultured in the presence of insulin. These results were not expected, based on the assumption
237 that *INS* mRNA expression decreased in insulin-cultured cells and that *PC1/3* and *PC2* are
238 essential for cleaving the proinsulin in insulin producing cells *in vivo* (67, 68). In human, the
239 mRNA for *PC1/3* and *PC2* are expressed in insulin producing cells in the pancreas (75),
240 which leads us to deduce that these enzymes would be upregulated in cells that express
241 pancreatic-related genes and insulin. An explanation for these results may be related with a
242 specific intestinal function of *PC1/3* and *PC2* expressed in duodenal cells. Additionally, the
243 *PC1/3* and *PC2* were already detected in rat small intestine (76) and it was observed that *PC2*
244 null mice exhibited gastrointestinal abnormalities and downregulation of several regulatory
245 peptides (77), becoming evident that these enzymes have specific functions in intestinal cells.

246 In a second experiment, we evaluated the expression of pancreatic-related genes in
247 duodenal cells cultured in the presence or absence of glucose and without insulin. Our
248 hypothesis was that intestinal-derived cells cultured in the presence of glucose would increase
249 the expression of pancreatic-related genes, once glucose is known to induce the increase in
250 insulin mRNA transcription, stability and secretion (78-83). Furthermore, high glucose

251 concentrations induce mesenchymal stem cells to differentiate into insulin producing cells
252 (84). Similarly, glucose associated to *Pdx1* expression induced human fetal liver cells to
253 differentiate in insulin producing cells (85). Surprisingly, independently of the presence or
254 absence of glucose, the mRNA expression of *INS* decreased in cells of the first passage;
255 however, a significant increase of this gene expression was observed in cells of second
256 passage. Glucose stimulation on insulin expression appears to be mediated by *Pdx1* (86-90)
257 and the expression of both *INS* and *Pdx1* is decreased in β-cells dysfunction or pancreatitis in
258 humans (91). In contrast, we observed that, *in vitro,* the mRNA expression for *Pdx1*, which
259 decreased at second passage, differing from those patterns detected for *INS*. Thus, the cells
260 seem to loss the capacity to maintain *Pdx1* expression levels in culture.

261 Epidermal growth factor has been proposed to be an important factor supporting the
262 expression of pancreatic transcript factors, as *Pdx1* (24) and *Ngn3* (92) *in vitro*. Our second
263 experiment was not designed to evaluate the effect of EGF, which was a component of the
264 culture medium, but it was possible to notice that the expression of *Pdx1* mRNA decreased in
265 intestinal cells cultured independently of the presence of glucose and EGF.

266 *NeuroD1* is another transcript factor important regarding regulation of insulin gene
267 transcription (93), mainly through the action in insulin gene enhancers, which appears to be
268 related to glucose interactions (64, 94). *NeuroD1* (95) and *Ngn3* (59-61) are endocrine
269 pancreatic progenitor markers. In the present study, we could observe that *NeuroD1* was
270 upregulated in the intestinal cultured cells from the second passage, either in the absence of
271 glucose and insulin or in the presence of glucose and the absence of insulin (Figure 2),
272 reaching higher levels of mRNA than those found in intestinal tissue before culture. Likewise,
273 the expression of *Ngn3* mRNA increased at second compared to first passage of cells and
274 reached similar levels of mRNA expression at second passage compared to those observed in
275 duodenal tissue. The expression of *Ngn3* and *NeuroD1* mRNA suggested that the piglet

276 duodenum has cells with characteristics of pancreatic endocrine progenitor cells. The
277 upregulation of these genes in cultured cells may indicate that the culture system without
278 insulin, which was used in the second experiment stimulated the expression of *Ngn3* and
279 *NeuroD1* genes. Concerning *PC1/3* and *PC2* genes, we found that the mRNA expression was
280 upregulated in the absence of insulin at second passage, indicating that the cells preserved the
281 expression capacity to cleave proinsulin in mature insulin (67, 68).

282 The mRNA expression obtained from intestinal cultured cells were encouraged, but
283 mature insulin was not detected either in newborn intestinal tissue or in cultured cells treated
284 with or without glucose. On the other hand, preceding studies showed that even though mice
285 duodenal cells have no mRNA expression for *INS*, these cells became able to produce insulin
286 after GLP1 treatment (96). Besides GLP1, other growth factors are involved in beta cell fate
287 and function, as fibroblastic growth factors (FGF) (41-44, 97-99), insulin-like growth factors
288 (IGF) (45), plateled-derived growth factors (PDGF) (100) and activin A (101, 102). Our data
289 indicated that the porcine intestinal cells have potential to produce insulin but probably
290 specific molecules or transcription factors are necessary for the final differentiation step.
291 Taken together, the results provide evidence that porcine intestinal cells have the machinery
292 to express *INS* mRNA and other genes related to pancreatic formation, development and
293 function, but not to produce insulin under our culture conditions. The results of this study
294 allowed us to infer that newborn piglet duodenal cells have potential to be transdifferentiated
295 in insulin producing cells.

296

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573 **List of Figures**

574 **Figure 1.** Expression of pancreatic related genes in piglet intestinal tissue (INT), cell
575 suspension before cell culture (CS) and cultured cells from first (P1) and second (P2)
576 passages. The cells were cultured with insulin (10 µg/ml) and without glucose. Different
577 letters above error bars indicate statistically significant differences between samples ($P<0.05$).
578 This experiment was performed in triplicate.

579

580 **Figure 2.** Expression of pancreatic related genes in piglet intestinal tissue (INT), cell
581 suspension before cell culture (CS) and cultured cells from first (P1) and second (P2)
582 passages. The cells were cultured in the absence (first - AP1 and second - AP2 passages) or in
583 the presence (25mM; first GluP1 and second GluP2 passages) of glucose. Different letters
584 above error bars indicate statistically significant differences between samples ($P<0.05$). This
585 experiment was performed in triplicate.

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587 **Figure 3.** Immunolocalization of insulin in adult (A, B and C) and newborn (D, E and F)
588 pancreas and newborn duodenal cells (G, H and I) by confocal laser scanning micrographs.
589 Immunofluorescence scale bars are 30 µm length and pictures were obtained at 400x
590 magnification. Morphology of cultured cells after passage 2 in the absence of both insulin and
591 glucose (J) and in the presence of 25mM of glucose (K). Images obtained with a Leica
592 Microscope DMI 4000B, equipped with Integrated Modulation Contrast (IMC; Leica
593 Microsystems) at 200x magnification.

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597 Table 1. List of primers used during gene expression experiments.

Gene	Forward primer	Reverse primer	GenBank or Ensembl accession number
*prepro*INS	GCAGAAGCGTGGCATCGT	GGCGGCCTAGTTGCAGTAGT	NM_001109772.1
PDX1	GCCTTTCCCGTGGATGAAG	GCCTAGAGATGTATTTGTTGAAAAGGA	ENSSSCT00000010200
NGN3	GCGAGCGCAATCGAATG	TTGAGTCAGCGCCCAGATG	NM_020999.3
NeuroD1	GGGCTCTGTCGGAGATCTTG	GCAGCCTGCAACCAGGTT	XM_003359578.1
PC1/3	GGGAGAGCTGATGGGAGGTT	GAGAACTTGGATCCTATGATCTGGAA	NM_214038.1
PC2	CTCTTTGGCTATGGAGTCCTTGA	GTACGGAGCCTCCCACACA	NM_001004044.1
B2M	CGCCCCAGATTGAAATTGAT	TCCACAGCGTTAGGAGTGAACTC	ENSSSCT00000005176

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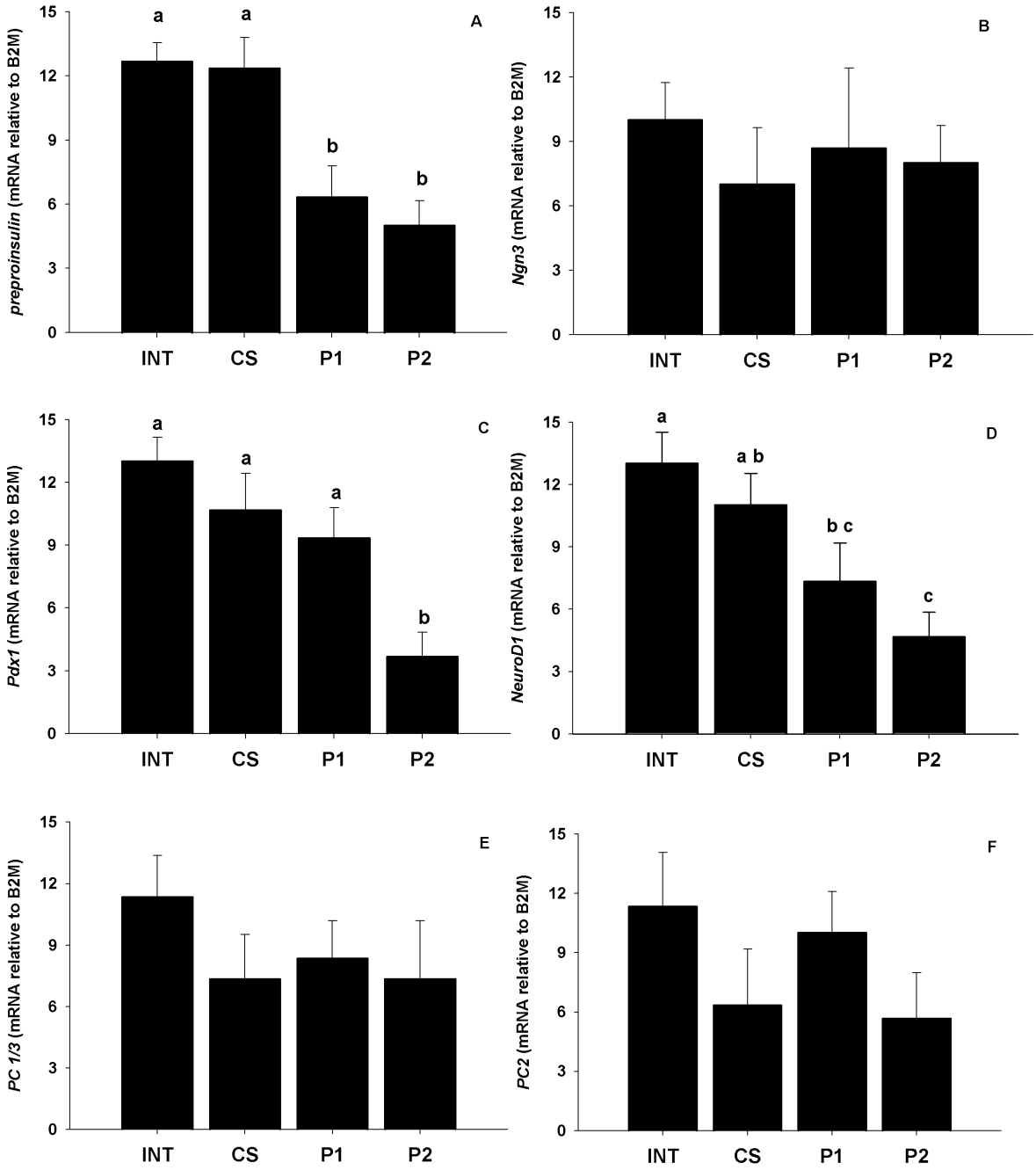
617 Figure 1.



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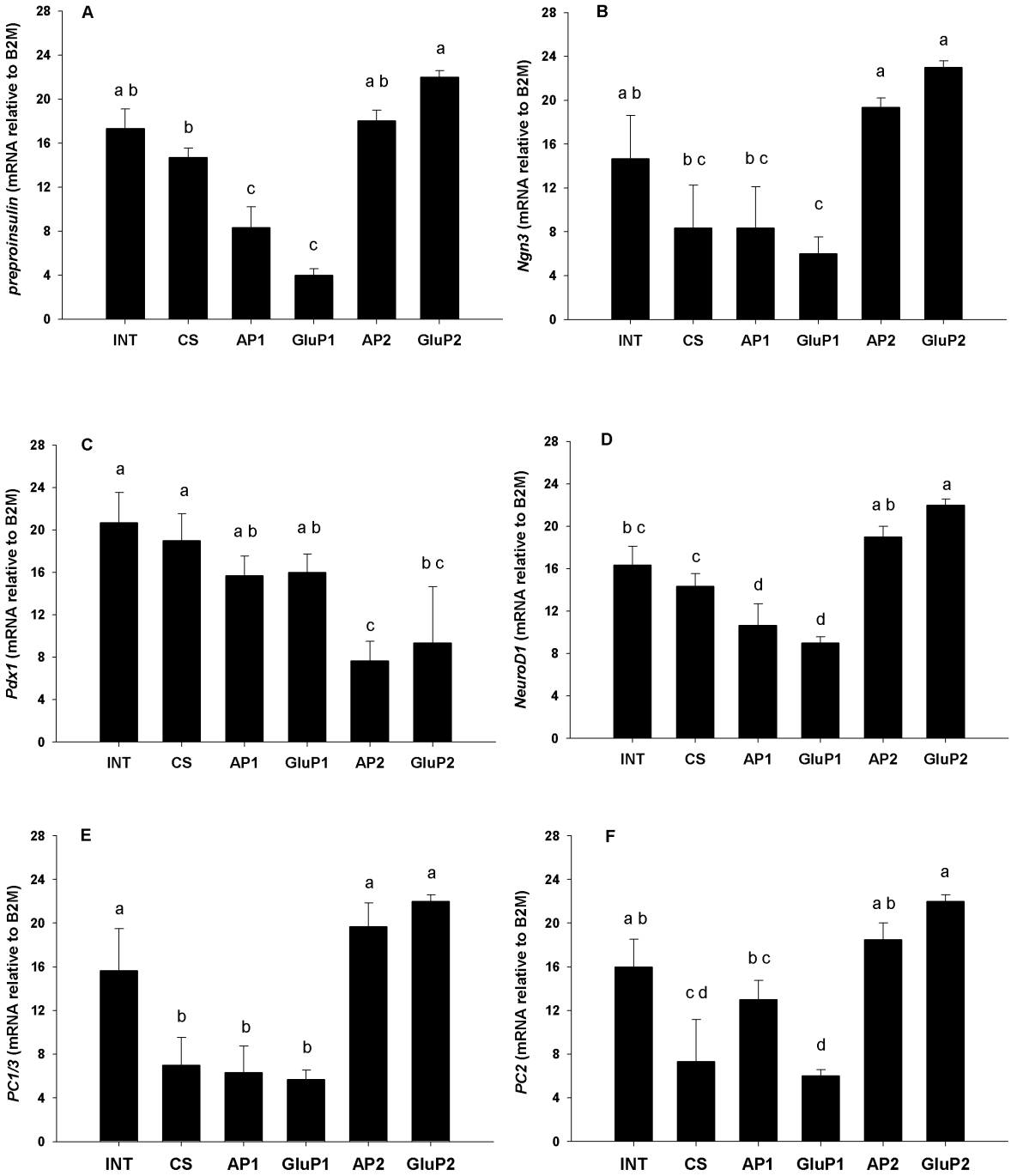
625 Figure 2.



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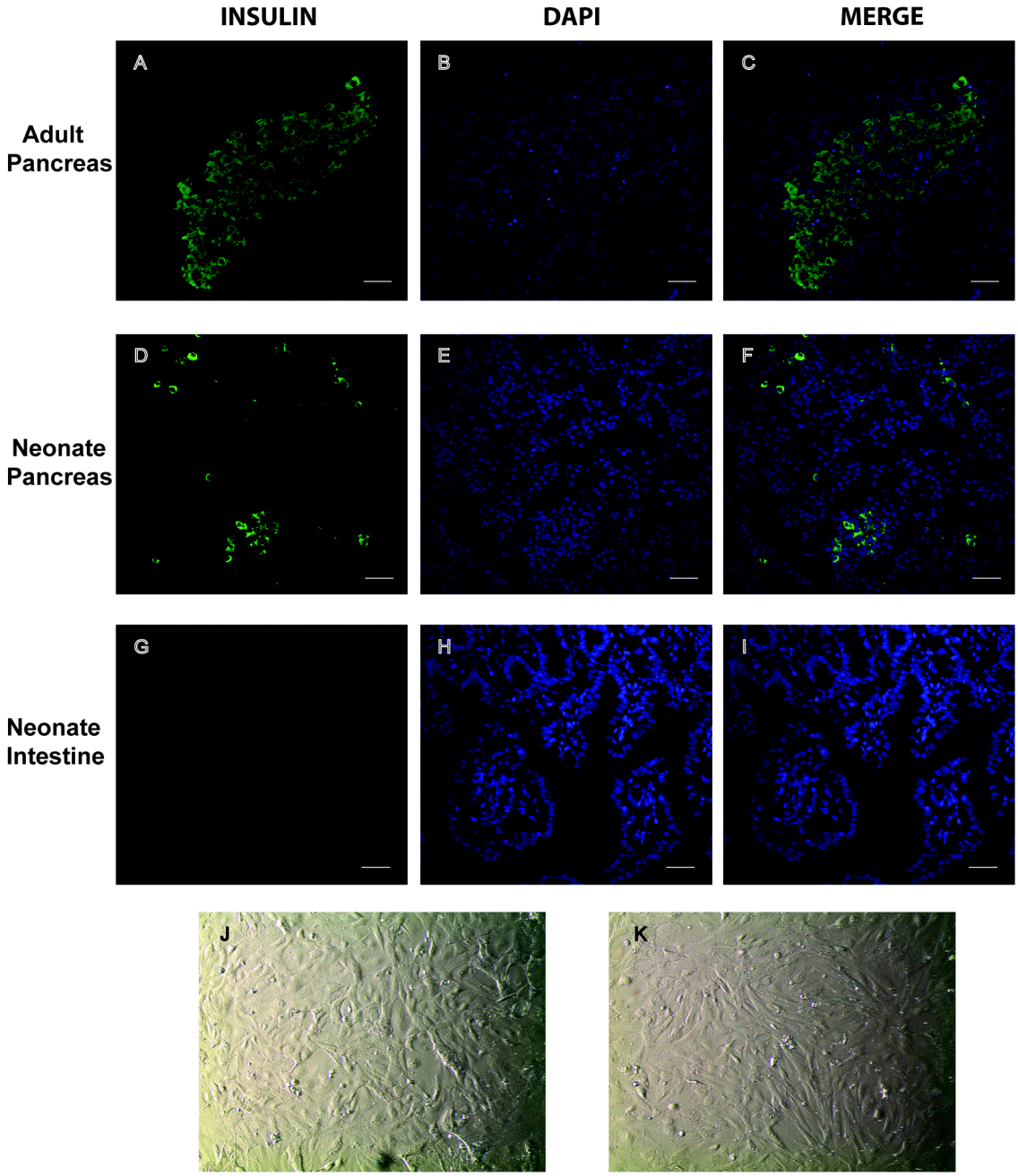
631 Figure 3.



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CONCLUSÃO

Com base nos resultados obtidos, conclui-se que células isoladas submetidas ao cultivo *in vitro* e tecidos oriundos do duodeno de suínos neonatos são capazes de expressar genes relacionados a formação, desenvolvimento e metabolismo da insulina. No entanto, as células intestinais antes e após o cultivo não são capazes de produzir insulina independente da presença ou ausência de glicose. Assim sendo, as células intestinais de suínos possuem a maquinaria molecular para produção de insulina; no entanto, estudos básicos são necessários para determinar quais os fatores estão ausentes para que as células se tornem produtoras de insulina responsiva à glicose para o tratamento do diabetes mellitus tipo 1.

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