

**UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS RURAIS
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA VETERINÁRIA**

**PROTEÍNAS LIGANTES DOS RECEPTORES DE
FATORES DE CRESCIMENTO EM COMPLEXO
CUMULUS-OÓCITO BOVINO**

DISSERTAÇÃO DE MESTRADO

Paulo Roberto Antunes da Rosa

Santa Maria, RS, Brasil

2011

**PROTEÍNAS LIGANTES DOS RECEPTORES DE FATORES
DE CRESCIMENTO EM COMPLEXO CUMULUS-OÓCITO
BOVINO**

Paulo Roberto Antunes da Rosa

Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Fisiopatologia da Reprodução, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Mestre em Medicina Veterinária.**

Orientador: Prof. Paulo Bayard Dias Gonçalves

**Santa Maria, RS, Brasil
2011**

**Universidade Federal de Santa Maria
Centro de Ciências Rurais
Programa de Pós-Graduação em Medicina Veterinária**

A Comissão Examinadora, abaixo assinada,
aprova a Dissertação de Mestrado

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CRESCIMENTO EM COMPLEXO CUMULUS-OÓCITO BOVINO**

elaborada por
Paulo Roberto Antunes da Rosa

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

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Santa Maria, 31 de agosto de 2011.

AGRADECIMENTOS

Aos meus queridos pais, Paulo Roberto Mariani da Rosa e Ambrozina Antunes da Rosa, por serem presença constante na minha vida fornecendo todo o apoio necessário, pelo amor, carinho e dedicação.

Aos demais membros da minha família, minha irmã Ana Cláudia, meu cunhado Augusto e minha namorada Camile, por todo apoio, carinho, amizade e compreensão.

Aos meus orientadores Paulo Bayard Dias Gonçalves e João Francisco Coelho de Oliveira, pela contribuição a minha formação profissional, pelos conhecimentos transmitidos e por serem exemplos de dedicação a ciência com base nos princípios éticos.

Ao laboratório BioRep, estagiários e colegas de Pós-Graduação, pela amizade, ajuda nos experimentos e principalmente por tornar o laboratório um ambiente familiar durante os dias de trabalho.

Ao Frigorífico Silva, por disponibilizar a coleta dos ovários utilizados nos experimentos.

Ao CNPq e CAPES pelo apoio financeiro.

RESUMO

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

PROTEÍNAS LIGANTES DOS RECEPTORES DE FATORES DE CRESCIMENTO EM COMPLEXO CUMULUS-OÓCITO BOVINO

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Data e Local da Defesa: Santa Maria, 31 de agosto de 2011.

O objetivo do presente trabalho foi caracterizar as proteínas Grb10 e Grb14 em complexos cumulus-oócito (CCOs) de bovinos oriundos de folículos em diferentes fases de desenvolvimento e mostrar o envolvimento do estradiol na regulação da expressão de RNAm. Primeiramente, foram obtidos pool de CCOs de folículos de 3-8mm para verificar a expressão de RNAm para Grb10 e Grb14 no oócito desnudo e nas células do cumulus. Tanto o oócito quanto as células do cumulus expressaram RNAm para as proteínas em estudo. Com o intuito de caracterizar o modelo experimental utilizado, foi verificado que a competência à progressão meiótica se dá em oócitos oriundos de folículos com diâmetro >2mm ($P<0,01$) e aumenta ao longo do desenvolvimento folicular, já que, oócitos oriundos de folículos de 1-3 e 4-6mm apresentam taxas de maturação inferiores aos oócitos oriundos de folículos de 6-8 e >8mm ($P<0,05$). O primeiro experimento foi delineado com o intuito de demonstrar uma expressão diferencial de mRNA para as proteínas Grb10 e Grb14 ao longo do desenvolvimento folicular. Para isso os grupos de CCOs (1-3, 4-6, 6-8 e >8mm) foram submetidos a extração RNA e transcrição reversa. A expressão relativa dos genes foi realizada por PCR em tempo real. Nesse experimento foi verificado que a expressão do gene Grb10 em CCOs esteve elevada nos grupos 1-3 e 4-6mm, diminuindo nos grupos 6-8 e >8mm ($P<0,05$). Já a expressão de mRNA de Grb14 esteve alta no grupo 1-3, diminuindo ($P<0,05$) conforme o aumento do tamanho folicular. Além disso, foi realizada a localização da proteína Grb10 pela técnica de imunofluorescência. A proteína Grb10 foi localizada tanto no oócito como no cumulus dos grupos de CCOs (1-3, 4-6, 6-8 e >8mm) porém a localização foi mais evidente em CCOs oriundos de folículos com diâmetro <6mm. Com o intuito de identificar uma influência hormonal na regulação da expressão de Grb10 e Grb14, foi realizado um terceiro experimento no qual CCOs oriundos de folículos de 3-8mm foram co-cultivados com metades foliculares em meio suplementado com estradiol-17 β e/ou fulvestrant (antagonista do estradiol) durante 6h. A expressão de RNAm não apresentou diferença significativa entre os tratamentos ($P>0,05$). Com base nesses resultados pode-se concluir que há expressão de RNAm para Grb10 e Grb14 bem como localização da proteína Grb10 em CCOs de bovinos, e que a expressão diferencial de RNAm sugere um envolvimento desses genes na aquisição de competência oocitária ao longo do desenvolvimento folicular. Portanto novos estudos precisam ser feitos para entender um mecanismo de regulação na expressão de RNAm.

Palavras chave: Grb10, Grb14, maturação, oócito, bovino.

ABSTRACT

Master's Dissertation

Programa de Pós-Graduação em Medicina Veterinária

Universidade Federal de Santa Maria

GROWTH FACTOR RECEPTOR-BOUND PROTEINS IN BOVINE CUMULUS-OOCYTE COMPLEX

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Date and Place of Defense: Santa Maria, August 31th, 2011.

The aim of this study was to characterize the Grb10 and Grb14 mRNA and protein expression in COCs derived from follicles of different stages of development (1-3, 4-6, 6-8 and >8mm in diameter) and determinate the involvement of the estradiol in the Grb10 and/or Grb14 mRNA expression. Firstly, a pool of the 80 COCs from follicles at 3 to 8mm was used to demonstrate Grb14 and Grb10 mRNA expression in denuded oocytes and respective cumulus cells. The expression was detected in oocyte and cumulus cells. To characterize the experimental model, oocytes from different follicle size were submitted to nuclear maturation assessment and evaluated for competence to reach metaphase II. Maturation competence began in oocytes from follicles larger than 2mm in diameter ($P < 0,01$) and increased throughout follicular development, since, oocytes from follicles of 1-3mm and 4-6mm had lower maturation rate than those from follicles of 6-8 and >8mm ($P < 0,05$). In the first experiment, Grb10 and Grb14 mRNA expression was assessed by qRT-PCR in COCs from different follicle size. The expression of Grb10 mRNA in oocytes from 1-3 and 4-6mm follicles was higher ($P < 0,05$) than those from 6-8 and >8mm follicles. However the Grb14 mRNA relative expression was higher in oocytes of group 1-3mm decreasing ($P < 0,05$) as the follicle size increase. In addition, we performed the localization of the Grb10 protein by immunofluorescence analysis. Positive fluorescence signal for Grb10 was detected in all analyzed samples. To evaluate an hormonal influence on the Grb10 and Grb14 mRNA expression, oocytes from follicles at 3-8mm were cultured in the presence of follicular cells in medium supplemented with 17β -estradiol and/or fulvestrant (an estradiol antagonist). The mRNA expression did not show statistical difference between treatments groups ($P > 0,05$). Based on this results, we conclude that Grb10 and Grb14 mRNA and Grb10 protein are expressed COCs in cattle. In addition, the differential expression observed in COCs from different follicle size suggest an involvement of the Grb10 and Grb14 genes in the bovine oocyte competence.

Key words: Grb10. Grb14. maturation oocyte. bovine.

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INTRODUÇÃO

Em mamíferos, o processo de maturação oocitária envolve três etapas, sendo elas a maturação citoplasmática, molecular e nuclear. A maturação oocitária *in vitro* tem sido alvo de estudos intensos uma vez que existe a participação de diversos fatores, que não têm suas funções estabelecidas até o momento. A importância de diversos fatores de crescimento no processo de maturação tem sido demonstrada, dentre eles os que possuem receptores do tipo tirosina quinase tais como: IGF-I (insulin-like growth factor-I), EGF (epidermal growth factor), FGF-10 (fibroblast growth factor 10) e VEGF (vascular endothelial growth factor) (SAKAGUCHI et al., 2000; EPPIG, 2001; HUNTER et al., 2004; ZHANG et al., 2010). A sinalização intracelular desencadeada pelos fatores supracitados é negativamente regulada em diversos tecidos pelas proteínas de ligação da família Grb-7, constituídas pelas proteínas Grb-7, Grb-10 e Grb-14. Essas duas últimas desempenham funções essenciais em regulações intracelulares relacionadas com proliferação e diferenciação celular (MORRIONE et al., 1997; MOUNIER et al., 2001) sendo foco de diversos estudos em doenças como tumores, diabetes e distúrbios de crescimento em humanos e camundongos (MCCANN et al., 2001; CHARALAMBOUS et al., 2003; GOENAGA et al., 2009b; HOLT et al., 2009). Além disso, estudos que utilizam células mamárias cancerígenas de mulheres como modelo experimental, apontam o estradiol como responsável por diminuir a expressão da proteína Grb14 e consequentemente aumentar a atividade proliferativa dessas células (KAIROUZ et al., 2005).

Na fisiologia da reprodução, estudos mostram uma participação do Grb14 no reinício da meiose de oócitos de xenopus (CAILLIAU et al., 2003; BROWAEYS-POLY et al., 2010). No entanto, as proteínas Grb-10 e Grb-14 ainda não foram estudadas em complexos cumulus-oócito (CCOs) de mamíferos. Dessa forma, na busca de conhecimento sobre os fatores que atuam no processo de maturação e aquisição de competência oocitária, essa dissertação visa caracterizar as proteínas Grb10 e Grb14 em CCOs de bovinos, partindo do princípio de que ocorre uma expressão diferencial de RNAm em CCOs oriundos de folículos em diferentes fases de desenvolvimento e que o estradiol, esteróide fundamental para o estabelecimento da dominância folicular, regula os níveis de expressão de RNAm para as proteínas em estudo.

Os experimentos foram delineados *in vitro*. O primeiro teve como objetivo demonstrar os níveis de expressão de RNAm para Grb10 e Grb14 em CCOs oriundos de folículos em diferentes fases de desenvolvimento (1-3, 4-6, 6-8 e >8mm de diâmetro). Concomitantemente a esse experimento, os grupos em estudo foram caracterizados quanto a localização da

proteína Grb10 nos diferentes compartimentos celulares dos grupos de CCOs anteriormente mencionados. O terceiro experimento foi delineado para responder se o estradiol regula a expressão de Grb10 e Grb14 em CCOs de bovinos. Para isso foi utilizado um modelo de maturação oocitária *in vitro* sob influência de estradiol e/ou fulvestrant (antagonista do receptor de estrógeno) nos meios de cultivo para posterior avaliação da expressão de RNAm para Grb10 e Grb14.

REVISÃO BIBLIOGRÁFICA

Maturação de oócitos

Maturação nuclear

Ao longo do desenvolvimento folicular, ocorre diversas modificações morfológicas e bioquímicas no oócito. Tais eventos, que iniciam com a formação do folículo primordial e continuam até o momento da ovulação, são responsáveis por tornar o oócito competente para o reinício da meiose, fecundação e, subsequente, desenvolvimento embrionário (BREVINI GANDOLFI & GANDOLFI, 2001). A maturação ocorre de maneira gradual e sincronizada com os eventos foliculares, visto que o desenvolvimento do folículo e seu oócito são eventos paralelos e relacionados funcionalmente (BEVERS et al., 1997).

Os oócitos são derivados das células germinativas primordiais, as quais, ainda no desenvolvimento fetal, transformam-se em oogônias que, por sua vez, diferenciam-se das outras células por apresentarem pouca quantidade de organelas e uma alta frequência de divisão mitótica, chegando a 2.700.000 oogônias no dia 110 de gestação em bovinos (ERICKSON, 1966). Porém esse número diminui, pois muitos oócitos sofrem processos degenerativos como resultados de erros genéticos ocorridos durante o “crossing over” ou devido a distúrbios metabólicos e/ou vasculares (MOTTA et al., 1997). Em torno dos 72-82 dias de gestação na vaca, alguns oócitos do feto já iniciam a primeira prófase meiótica, passando então pelos estádios de leptóteno, zigóteno, paquíteno e diplóteno, onde ocorre a primeira parada da meiose, também denominado estágio de dictióteno ou de vesícula germinativa (VG; RICHARDS, 1980).

A maturação final do oócito, com o objetivo de produzir um oócito haplóide, ocorre no folículo ovulatório, após o pico de LH (hormônio luteinizante). O reinício da meiose ocorre pela dissolução da membrana nuclear e condensação da cromatina no processo denominado de rompimento da vesícula germinativa (RVG), e a maturação nuclear passa pelos estádios de metáfase I (MI), anáfase I (AI), telófase I (TI) e progride até a metáfase II (MII), onde ocorre a segunda parada da meiose (GORDON, 1994).

A retirada do oócito do interior do folículo para maturação *in vitro* também é fator determinante para o reinício da meiose e posterior rompimento da vesícula germinativa (GVBD), uma vez que, segundo LEIBFRIED and FIRST (1979), as células foliculares produzem fatores que inibem a progressão meiótica. O tempo requerido para a maturação

nuclear varia dependendo da espécie. No bovino, a RVG ocorre de 6-12 horas, a MI de 12-15 horas, a AI e a TI de 15-18 horas e a MII de 18-22 horas após o pico de LH ou após a retirada do oócito do folículo (SIRARD et al., 1989; WU et al., 1997).

O processo de competência oocitária é definido como sendo a capacidade do oócito em completar a maturação, ser fertilizado e sustentar o desenvolvimento embrionário subsequente (BREVINI GANDOLFI & GANDOLFI, 2001). A competência é adquirida gradualmente e ao longo do desenvolvimento folicular. Em bovinos, existe um consenso geral que para o oócito adquirir competência satisfatória o folículo deve ter no mínimo 2 a 3 mm de diâmetro, e que folículos de maior diâmetro contém oócitos com maior potencial de desenvolvimento (PAVLOK et al., 1992; ARLOTTO et al., 1996; MACHATKOVA et al., 2004; LEQUARRE et al., 2005).

Nos bovinos, uma complexa cascata de eventos de fosforilação e desfosforilação está envolvida na regulação do reinício da meiose. Uma proteína de 79kD conhecida como fator promotor da maturação (MPF) é responsável pelo início da maturação nuclear do oócito e sua ativação precede ou ocorre concomitantemente com o rompimento da vesícula germinativa (WU et al., 1997). O fator promotor da maturação é um complexo formado por uma subunidade regulatória (45kD) conhecida como ciclina B e uma subunidade catalítica de 34kD (p34Cdc2 kinase) (GAUTIER et al., 1990). A ativação do MPF induz a condensação cromossomal, rompimento do envelope nuclear e reorganização citoplasmática com posterior entrada na fase-M de ambos ciclos celulares meiótico e mitótico (MOTLIK & KUBELKA, 1990). Durante o crescimento dos oócitos, a síntese e o acúmulo de p34Cdc2 parecem ser necessários para a aquisição de competência ao rompimento da vesícula germinativa e progressão meiótica (CHESNEL & EPPIG, 1995).

Durante o processo de maturação do oócito em mamíferos, existem evidências de que alguns eventos sejam regulados pela MAPK (mitogen-activated protein kinase). Em oócitos de mamíferos estão presentes duas isoformas da MAPK conhecidas como extracellular regulated kinase [ERK1(p44)] e [ERK2(p42)] (FAN et al., 2002). A Ativação da MAPK é desencadeada pela fosforilação de resíduos de tirosina e treonina e é fundamental para a progressão da meiose de oócitos de diferentes espécies. O momento exato da ativação da MAPK não está determinado, porém estudos sugerem que, em bovinos, o aumento nas atividades do MPF e da MAPK são considerados necessários para o rompimento da vesícula germinativa e progressão da metáfase (FAN & SUN, 2004; SUN et al., 2009). Em outro estudo, foi demonstrado que a MAPK é ativada no estágio de RVG, tendo sua atividade máxima em MI, permanecendo elevada até a formação dos pronúcleos (FISSORE et al.,

1996). A atividade da MAPK é essencial para a manutenção do MPF, formação dos fusos meióticos e manutenção do bloqueio meiótico na fase de MII (COLLEDGE et al., 1994; HASHIMOTO et al., 1994).

Maturação citoplasmática e molecular

Durante o desenvolvimento folicular, os oócitos acumulam RNAm e proteínas de origem materna que são fundamentais para suportar o desenvolvimento embrionário precoce, antes da ativação do genoma embrionário (SIRARD, 2001). No oócito, ao contrário do que ocorre com qualquer célula somática, o intervalo entre a síntese e a utilização do RNAm e moléculas protéicas pode ser de até várias semanas, ocorrendo o armazenamento dessas moléculas em uma forma quiescente para o seu emprego no tempo certo ao longo da maturação do oócito e desenvolvimento embrionário inicial (HYTTEL et al., 1997). A presença de RNAm que codifica para proteínas específicas é que determina a qualidade do oócito e, finalmente, a sua competência para sustentar o desenvolvimento embrionário (BREVINI GANDOLFI & GANDOLFI, 2001).

Após o pico do hormônio LH *in vivo* ou, após a remoção do oócito do ambiente folicular para posterior maturação *in vitro*, a atividade de transcrição é drasticamente diminuída e o oócito já é capaz de reiniciar a meiose e atingir a metáfase II. Provavelmente, esse é um sinal comum para ativar o MPF e a MAPK para a maturação dos oócitos nos mamíferos (DEKEL, 1996).

Durante a maturação citoplasmática do oócito ocorre o rearranjo bem como um aumento no número de organelas citoplasmáticas. Dentre estas organelas, as mitocôndrias desempenham função importante, uma vez que sintetizam o ATP necessário para a síntese de proteínas fundamentais para os processos de maturação e desenvolvimento embrionário subsequentes (STOJKOVIC et al., 2001).

Os ribossomos, organelas produzidas no nucléolo da célula, atuam na síntese de proteínas no ooplasma. Desta forma, oócitos em estágios iniciais da primeira divisão meiótica e embriões em fase de ativação do genoma apresentam elevada atividade de síntese ribossomal refletindo em uma alta atividade de síntese protéica, porém em oócitos oriundos de folículos primordiais ocorre ausência de atividade de síntese ribossomal e conseqüentemente uma baixa atividade de síntese protéica (HYTTEL et al., 2001).

A maturação citoplasmática do oócito começa nos folículos primordiais, onde as modificações no oócito e no ambiente folicular são eventos paralelos e dependentes entre si

para que ocorra o desenvolvimento do folículo dominante com posterior ovulação de um gameta fértil e capaz de sustentar o desenvolvimento embrionário (CAMPBELL & MCNEILLY, 1996). Oócitos competentes ao reinício da meiose e progressão até o estágio de metáfase II devem possuir um diâmetro $>110\mu\text{m}$, sendo que este tamanho só é atingido quando o folículo de origem atinge um diâmetro de 3mm (FAIR et al., 1995). As taxas de blastocistos obtidas com a fecundação *in vitro* de oócitos oriundos de folículos pequenos é inferior quando comparada a fecundação de oócitos oriundos de folículos maiores (YANG et al., 1998b; LEQUARRE et al., 2005).

As principais modificações observadas durante a fase de desenvolvimento do oócito são: a) formação das junções intercomunicantes, b) desenvolvimento e deslocamento do complexo de Golgi para a periferia do oócito, c) desenvolvimento do retículo endoplasmático liso e das gotas de lipídio, d) formação dos grânulos corticais e zona pelúcida, e) diferenciação e rearranjo das mitocôndrias no ooplasma, f) quebra dos centríolos, g) transcrição de mRNA materno para a síntese de proteínas importantes para o oócito e subsequente desenvolvimento embrionário (HYTTEL et al., 1997).

Além das gonadotrofinas LH e FSH, fundamentais durante a foliculogênese e maturação oocitária, há também o envolvimento de outros tipos de sinalizações no ambiente folicular denominadas sinalizações parácrinas e autócrinas que ocorrem através da liberação de fatores de crescimento sintetizados tanto pelos oócitos quanto pelas células da granulosa. Dentre esses fatores podemos citar: BMP-15 (bone-morphogenetic factor-15), GDF-9 (growth-differentiation factor-9), FGF-2 (fibroblast growth factor-2), FGF-10, IGF-1, EGF e VEGF (EPPIG, 2001; HUNTER et al., 2004; SAGIRKAYA et al., 2007; HSIEH et al., 2009; CHAVES et al., 2010; NISHIGAKI et al., 2010).

Alguns desses fatores têm sido estudados durante o processo de maturação. Estudos evidenciam uma participação do IGF-I no processo de maturação oocitária. A adição de IGF-I no meio de maturação *in vitro* acelera a progressão da meiose pelo possível aumento nas atividades da histona H1 e da MAPK durante as fases iniciais da maturação nuclear de oócitos bovinos (SAKAGUCHI et al., 2002). Além disso, a adição de IGF-I ao meio de maturação aumenta o número de embriões que chegam ao estágio de blastocisto (HERRLER et al., 1992). Alguns estudos realizados em diferentes espécies demonstram que o EGF, quando utilizado no meio de maturação *in vitro*, contribui para um aumento nas taxas de blastocistos (LONERGAN et al., 1996; ILLERA et al., 1998; PUROHIT et al., 2005). Além do IGF-I e do EGF atuando no processo de maturação oocitária, estudos recentes mostram o envolvimento do FGF-10 no processo de maturação de oócitos bovinos, uma vez que, quando adicionado no

meio de maturação oocitária promove um aumento na progressão da meiose bem como nas taxas de blastocistos (ZHANG et al., 2010).

Da mesma forma que os fatores supracitados, a insulina juntamente com seus receptores, têm se mostrado importante para os eventos relacionados à reprodução. Na espécie bovina, demonstrou-se que a insulina, quando adicionada ao meio de maturação *in vitro*, acelera a progressão da meiose e tem efeito positivo na clivagem embrionária (BORTOLOTTO et al., 2001). Além disso, quando adicionada ao meio de cultivo, a insulina têm aumentado os índices de desenvolvimento embrionário (MATSUI et al., 1997; AUGUSTIN et al., 2003).

Proteínas Grb-10 e Grb-14

As proteínas Grb10 e Grb14 juntamente com o Grb7, formam as proteínas de ligação da família Grb7. Essas proteínas são bastante estudadas na espécie humana, onde possuem grande importância regulando os receptores do tipo tirosina quinase, tais como os receptores do IGF-I, FGF, EGF, VEGF. A maioria das pesquisas com a espécie humana estuda a participação dessas proteínas no desenvolvimento de tumores responsivos ao IGF-I, metabolismo da insulina em diabetes e anormalidades de crescimento (MCCANN et al., 2001; LIM et al., 2004; DI PAOLA et al., 2006).

Em humanos, o gene do Grb7, Grb10 e Grb14 estão localizados nos cromossomas 17, 7 e 2, respectivamente. Enquanto que nos bovinos essa localização se dá, na mesma ordem, nos cromossomas 19, 4 e 2 (LUCAS-FERNANDEZ et al., 2008). As proteínas da família Grb7 são encontradas em vários tecidos que possuem receptores do tipo tirosina quinase. A expressão do Grb7 já foi descrita no pâncreas, fígado, intestino e próstata, enquanto o Grb10 e Grb14 já foram encontrados em músculo esquelético, tecido adiposo, coração, rins, cérebro, fígado, intestino, placenta, testículos e ovários. Além disso, são muito expressos em tecidos fetais. O papel dessas proteínas não está claro, mas estudos indicam uma participação das mesmas na regulação da migração celular, angiogênese, metabolismo celular, desenvolvimento embrionário e proliferação celular (HAN et al., 2001).

Em humanos, as proteínas da família Grb7 são formadas pelos domínios SH2 (*Src homology 2*), PH (*pleckstrin homology*), BPS (*between the PH and SH2 domains*), P (*Proline-rich region*) e RA (*Ras-association*), possuindo uma grande homologia entre os diferentes membros dessa família (HAN et al., 2001). A subunidade SH2 do Grb10 quando comparada ao Grb7 e Grb14 possui 67% e 72% de homologia respectivamente, a porção BPS do Grb10

possui uma homologia de 56% e 64% ao Grb7 e Grb14 respectivamente, enquanto a porção PH do Grb10 possui uma homologia de 61% e 62% ao Grb7 e Grb14 respectivamente (HOLT & SIDDLE, 2005).

Apesar dessa homologia, essas proteínas possuem funções distintas no controle de diferentes processos fisiológicos. O Grb7 está relacionado com um controle da migração celular e angiogênese, o Grb10 controla o metabolismo celular, proliferação celular e o desenvolvimento embrionário, enquanto o Grb14 está relacionado com a proliferação e metabolismo celular (LUCAS-FERNANDEZ et al., 2008).

Grb10 é um gene imprinted, ou seja, somente um dos alelos (paterno ou materno) é responsável pela expressão do Grb10 nos diferentes tecidos. Estudos demonstram que o alelo materno é o responsável pela expressão do Grb10 uma vez que a sua deleção em camundongos resulta em crescimento acelerado dos embriões bem como da placenta. Além disso, foi observado que a deleção do alelo materno, e não do alelo paterno, contribui significativamente para a diminuição da expressão de Grb10 na placenta (CHARALAMBOUS et al., 2010).

As proteínas da família Grb7 estão localizadas no citoplasma celular, na forma inativa, após a ativação dos receptores tirosina quinase ocorre a migração dessas proteínas para a membrana plasmática da célula, aonde se ligam a sítios fosforilados desses receptores (NANTEL et al., 1999). Quando essas proteínas estão em sua forma inativa no citoplasma celular, elas se encontram na forma oligomerizada, ou seja, formando tetrâmeros. Esses tetrâmeros são formados pela ligação das subunidades SH2 e PH de uma proteína a região N terminal de outra. O sinal que desencadeia a dissociação dos tetrâmeros, forma inativa, em monômeros, forma ativa, é a ativação dos receptores tirosina quinase (DONG et al., 1998).

A ativação dos receptores tirosina quinase ocorre pela ligação de fatores de crescimento na porção extracelular desses receptores que é conhecida como subunidade α . A partir dessa ligação, ocorre ativação dos receptores tirosina quinase e subsequente fosforilação de resíduos de tirosina em sítios específicos da subunidade β desses receptores, os quais se encontram na porção intracelular. Com isso, ocorre a migração de substratos dos receptores de insulina (IRS-1 e IRS-2) e do Shc (Src homology collagen) que se ligam aos sítios fosforilados dos receptores tirosina quinase. Esses mediadores intracelulares, IRS e Shc, ativam as rotas do PI3K (phosphatidylinositol 3-kinase) e da MAPK (Mitogen-activated protein kinase), respectivamente, e com isso vão desencadear mudanças intracelulares na expressão de genes relacionados com o controle do metabolismo e proliferação celular (YOUNGREN, 2007).

Em células que possuem as proteínas da família Grb7, no momento da ativação dos receptores tirosina quinase, essas proteínas se ligam através das subunidades SH2 e BPS aos sítios fosforilados da porção β destes receptores. Essa ligação resulta no impedimento do acesso do IRS e do Shc aos sítios fosforilados dos receptores tirosina quinase. Com isso, resultando no bloqueio da ativação das rotas intracelulares do PI3K e da MAPK (LIM et al., 2004).

Estudos em humanos e camundongos demonstram que as proteínas Grb10 e Grb14 estão envolvidas no crescimento normal de células somáticas através da inibição da sinalização intracelular desencadeada pelo IGF-I e insulina. CHARALAMBOUS et al. (2003) utilizando um modelo experimental de silenciamento da expressão do gene Grb10 em camundongos, mostraram que a prole desses camundongos nulos para o gene Grb10 apresentavam crescimento embrionário acelerado. Em outro estudo utilizando a técnica de RNA de interferência em uma linhagem de células humanas responsivas ao IGF-I, foi demonstrado que a inibição do Grb10 no meio intracelular provoca um aumento na fosforilação do receptor de IGF bem como ativação da rotas Akt/PKB e ERK1/2 necessárias para o crescimento e diferenciação das células.

Estudos em humanos demonstram que células de câncer de mama apresentam uma inibição da atividade proliferativa quando tratadas com um inibidor do estrógeno. Essa inibição é ocasionada principalmente pelo bloqueio de rotas intracelulares ativadas pelo IGF-I (FREISS et al., 1998). Devido a importância das proteínas da família Grb7 em regular negativamente os receptores de IGF-I, Kairouz et al., (2005) estudaram o papel do estradiol na expressão de Grb14 em células mamárias cancerígenas de mulheres. No cultivo *in vitro* utilizado o estradiol diminuiu a expressão da proteína Grb14, além disso, foi verificado um aumento do Grb14 com a utilização de um inibidor de estrógeno específico ao cultivo celular. Analisando esses estudos, pode-se concluir que o estradiol atua no processo de proliferação das células de câncer de mama promovendo a inibição da proteína Grb14 e com isso, aumentando a sinalização intracelular desencadeada pelo IGF-I.

Existem estudos em xenopus demonstrando o envolvimento das proteínas da família Grb7 na fisiologia reprodutiva (CAILLIAU et al., 2003; GOENAGA et al., 2009a; BROWAEYS-POLY et al., 2010). Cailliau et al.(2003) demonstraram através de um modelo experimental, *in vitro*, utilizando oócitos de Xenopus expressando receptores de FGF, que a proteína Grb14 possui a capacidade de inibir o reinício da meiose quando esses oócitos são estimulados por FGF. Mostrando com isso, que o Grb14 regula negativamente os receptores de FGF. Cailliau et al., (2003) também demonstrou que as rotas intracelulares responsáveis

pelo bloqueio da maturação, causada pela proteína Grb14, são as rotas PI3K (pelo bloqueio da fosforilação do Akt) e MAPK (pelo bloqueio da fosforilação da ERK e do Raf).

ARTIGO 1

TRABALHO A SER ENVIADO PARA PUBLICAÇÃO:

GROWTH FACTOR RECEPTOR-BOUND PROTEIN 14: A POTENTIAL NEW GENE ASSOCIATED WITH OOCYTE COMPETENCE

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ZYGOTE, 2011

Growth factor receptor-bound protein 14: a potential new gene associated with oocyte competence

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Running Title: Grb14 in cumulus-oocyte complexes

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1 **Summary**

2 The Grb14 protein is a member of the Grb7 protein family. This protein family
3 acts by binding to tyrosine kinase receptors, promoting cell proliferation and
4 differentiation. There are evidences of the involvement tyrosine kinase factors in
5 the bovine oocyte maturation process. However, the Grb14 has not been studied
6 on bovine cumulus-oocyte complex (COCs). The aim of the present study was to
7 characterize the Grb14 mRNA expression in bovine COCs during follicular
8 development. Furthermore, we demonstrated that the expression of Grb14 mRNA
9 is not regulated by estradiol. The mRNA expression of Grb14 was assessed in a
10 total of 480 COCs from follicles of different size (1-3, 4-6, 6-8 and >8mm) by qRT-
11 PCR. The Grb14 mRNA expression decreased in COCs through the follicular
12 growth ($p<0.05$). The role of estradiol was studied in the expression of Grb14
13 mRNA in COCs. The Grb14 mRNA abundance did not differ in COCs cultured in
14 presence or absence of 17β -estradiol or fulvestrant. In conclusion, we showed that
15 Grb14 mRNA is downregulated in COCs during antral follicle development,
16 suggesting a role of Grb14 in oocyte competence.

17 **Key words:** Grb14, oocyte maturation, follicle development, oocyte, cattle

18

19 **Introduction**

20 Growth factor receptor-bound protein 14 (Grb14) is a member of adaptor
21 proteins superfamily that includes Grb7 and Grb10. This family of proteins has a
22 similar structure and a conservative molecular architecture consisting of an N-

23 terminal proline rich region, a central pleckstrin homology (PH), a carboxyl-
24 terminal SH2 domain, followed by a domain between the PH and SH2 domains
25 termed the BPS region. The structure of the Grb7 family binding proteins allow
26 the participation of these proteins in multiples cellular signal transduction
27 pathways (Lucas-Fernandez *et al.*, 2008).

28 The Grb7 protein family is involved in metabolic processes of cells, triggered
29 by growth factors that bind to tyrosine kinase receptors such as insulin-like
30 growth factor I (IGF-I) and epidermal growth factor (EGF; Holt and Siddle, 2005).
31 Upon tyrosine kinase receptors activation, the Grb14 binds to phosphorylated
32 sites of the β portion of these receptors through SH2 and BPS subunits. This
33 binding prevents the access of receptor substrates to the phosphorylated sites of
34 tyrosine kinase receptors, resulting in blocking the activation of the intracellular
35 pathways of mitogen-activated protein kinase (MAPK) and phosphatidylinositol-
36 3 kinase (PI3K). The MAPK and PI3K, are important for cell proliferation and
37 differentiation (Han *et al.*, 2001).

38 The Grb7 family of proteins have been related with IGF-I-dependent tumors
39 (Lim *et al.*, 2004), insulin metabolism (Goenaga *et al.*, 2009), diabetes (Holt *et al.*,
40 2009) and growth abnormalities (Charalambous *et al.*, 2003; McCann *et al.*, 2001).
41 In human breast cancer cells, the expression of Grb14 decrease when estradiol was
42 added to the cell culture *in vitro* (Kairouz *et al.*, 2005). In the physiology of
43 reproduction, reports in xenopus oocytes showed that Grb14 inhibited germinal
44 vesicle breakdown (GVBD) acting through tyrosine kinase receptors (Browaeys-

45 Poly *et al.*, 2010; Cailliau *et al.*, 2003; Goenaga *et al.*, 2009). To our knowledge,
46 Grb14 protein has not been studied on mammalian oocyte.

47 In most mammals, the follicle-enclosed oocyte is arrested at the diplotene
48 stage of the first meiotic prophase. In cattle, oocytes develop competence to
49 undergo GVBD when the follicle reaches 2-3 mm in diameter (Arlotto *et al.*, 1996;
50 Lequarre *et al.*, 2005). The oocytes undergo meiotic resumption after the
51 preovulatory LH surge *in vivo* or, spontaneously, after oocyte removal from the
52 follicular environment *in vitro* (Sirard *et al.*, 1989; Wu *et al.*, 1997). However, the
53 resumption of meiosis is delayed when the oocytes are maintained with follicular
54 hemisections *in vitro* (Richard and Sirard, 1996).

55 Cytoplasmic and molecular oocyte maturation is an event that occurs
56 simultaneously with the increase in the follicular diameter, secretion of estradiol
57 and establishment of dominance in mammals (Assey *et al.*, 1994; Campbell and
58 McNeilly, 1996; Yang *et al.*, 1998). These changes in the follicular environment are
59 caused by follicular activity of the gonadotrophic hormones (LH and FSH) and
60 growth factors that act through tyrosine kinase receptors such as IGF-I and EGF
61 (Reizel *et al.*, 2010; Rivera and Fortune, 2003). In cattle, the higher concentration of
62 the 17 β -estradiol in larger follicles than in small follicles may be used for
63 differentiation between dominant and atretic follicles (Meidan *et al.*, 1993).

64 Several mechanisms involved in the acquisition of developmental competence
65 by the oocyte are not yet well understood. *In vitro* studies in different species
66 showed that growth factors, which act through tyrosine kinase receptors, increase
67 the maturation rates and blastocyst development (Li *et al.*, 2004; Purohit *et al.*,

68 2005; Wang et al., 2009; Zhang et al., 2010). The intracellular routes activated by
69 these growth factors are not fully understood, but there are evidences that they
70 activate signal transduction pathways, such as MAPK and PI3K, to promote cell
71 proliferation and differentiation (Sakaguchi et al., 2002; Sun et al., 2009). The
72 MAPK activation is an important intracellular signaling event during bovine
73 oocyte maturation, necessary for the GVBD and metaphase progression (Fissore *et*
74 *al.*, 1996; Nurse, 1990).

75 Therefore, we hypothesized that Grb14 mRNA needs to be downregulated in
76 COCs by estradiol during antral follicle development for the oocyte to acquire
77 competence. The present study characterizes the expression of Grb14 mRNA in
78 COCs from different ovarian follicular diameter. Furthermore, we demonstrated
79 that the expression of Grb14 mRNA is not regulated by estradiol.

80

81 **Materials and Methods**

82 All chemicals used were purchased from Sigma Chemicals Company, St.
83 Louis, MO, USA, unless otherwise indicated in the text.

84

85 **Cumulus-oocyte complexes for qRT-PCR**

86 Abattoir ovaries were transported to the laboratory in saline solution (0.9%
87 NaCl) cooled (4°C) with 100IU/ml penicillin and 50µg/ml streptomycin stored in
88 a thermal box. In the laboratory, follicles (1-3, 4-6, 6-8 and >8mm in diameter)
89 were aspirated with a vacuum pump (vacuum rate of 20ml/minute). The COCs
90 were recovered and selected (grade 1 and 2) under a stereomicroscope according

91 to Leibfried and First (1979). In three replicates, a total of 480 COCs grade 1 and 2
92 (mean of 40 per group) were washed three times with PBS and placed into a 200 μ l
93 Trizol (Invitrogen, Carlsbad, CA, USA) for total RNA extraction and further study
94 of the Grb14 gene expression by qRT-PCR assay.

95

96 **Total RNA extraction and qRT-PCR**

97 Total RNA was extracted from samples of 30 to 50 COCs using Trizol
98 according to the manufacturer's instructions. Total RNA was quantified by
99 absorbance at 260nm using a NanoDrop 1000 spectrophotometer (Thermo
100 Scientific) and RNA integrity was verified electrophoretically on 1% agarose gel
101 stained by ethidium bromide. The purity was obtained by absorption rate
102 relationship OD260/OD280. Values <1.7 were not used in this study.

103 Total RNA was treated with DNase (Promega, Madison, WI) at 37°C for 5min
104 and 65°C for 10min to digest any contaminating DNA. The transcriptase reverse
105 reaction was performed with 1 μ M oligo-dT primer, 4U omniscrypt RTase
106 (Omniscrypt RT Kit, Qiagen, Mississauga, Canada), 0.5mM dNTP's and 10U
107 RNase inhibitor (Amersham Biosciences, São Paulo, Brasil). The relative gene
108 expression was performed by qRT-PCR using the StepOnePlus™ RT-PCR system
109 (Applied Biosystems, Foster City, CA) and variability in the amount of mRNA
110 was corrected by amplification of housekeeping gene GAPDH. The calculation of
111 relative expression was performed as recommended by Pfaffl (2001). The primers
112 of Grb14 (F- TCCCCAAAGCAAATTCAAGG e R-
113 AGGATCAACAGCTGGCAAAC) and housekeeping GAPDH (F-

114 GATTGTCAGCAATGCCTCCT e R- GGTCATAAGTCCCTCCACGA) was
115 designed in Primer Express Software v 3.3 (Applied Biosystems) and synthesized
116 by invitrogen for quantitative analysis of the abundance of Grb14 mRNA.

117

118 **Assessment of oocyte nuclear maturation to validate the experimental model**

119 Ovaries obtained from abattoir were transported to the laboratory in saline
120 solution (0.9% NaCl) at 30°C containing 100IU/ml penicillin and 50µg/ml
121 streptomycin stored in a thermal box. In the laboratory, the ovaries were washed
122 three times with saline (pre-heated to 30°C). COCs were aspirated from follicles at
123 1-2 and 3-8mm in diameter. Then, COCs were recovered and selected as described
124 above. After recovered, the COCs were randomly transferred into 4-well culture
125 plates (Nunc®, Roskild, Denmark) containing 400µl of the maturation medium
126 consisted by TCM-199 with Earle's salts, L-glutamine, Hepes and sodium
127 bicarbonate (Gibco Labs, Grand Island, NY, USA) supplemented with 100UI/ml
128 penicillin, 50µg/ml streptomycin, 5µg/ml LH (Lutropin-V®, Bioniche, Ontario,
129 CA), 0.5g/ml FSH (Folltropin®-V, Bioniche, Ontari, CA), 0.2mM sodium pyruvate
130 and 0.4%BSA. COCs were submitted to culture in an incubator at 39°C, an
131 atmosphere of 5%CO₂ in air and saturated humidity as described by Stefanello et
132 al. (2006). After 20h of culture in maturation medium, the cumulus cells were
133 removed by vortexing and oocytes were fixed in paraformaldehyde 4% during
134 15min and than were transferred 0.5% Triton X-100. Oocytes were stained with
135 10µg/ml bisbenzimidazole (Hoechst 33342) and analyzed under a fluorescence
136 microscope. Oocytes assessment were classified according to the nuclear

137 maturation stage as immature: germinal vesicle (GV), GVBD, metaphase I (MI)
138 and matured: anaphase I (AI), telophase I (TI), and metaphase II (M-II).

139

140 **The estradiol effect on Grb14 mRNA expression**

141 Grade 1 and 2 COCs were recovered and selected as described above. After
142 recovered, the COCs were randomly distributed into 4-well culture plates
143 containing 200µl of maturation droplets and cultured under the same conditions
144 described above during 6h. The maturation medium used was TCM-199 with
145 Earle's salts, L-glutamine, Hepes and sodium bicarbonate (Gibco Labs, Grand
146 Island, NY, USA) supplemented with 100UI/ml penicillin, 50µg/ml streptomycin,
147 0.5g/ml FSH (Folltropin®-V, Bioniche, Ontario, CA), 0.2mM sodium pyruvate and
148 0.4%BSA. The COCs were matured in the presence of two follicular hemisections
149 per 50µl of maturation medium (Richard and Sirard, 1996), in the presence or
150 absence of 500ng/ml 17β-estradiol (E2) and/or 100µM fulvestrant (Fulv; an
151 estrogen receptor antagonist). These concentrations were chosen based on
152 previous studies (Ali and Sirard, 2002; Luo and Wiltbank, 2006; Wang *et al.*, 2006).
153 After culture for 6h, COCs were recovered, placed into 200µl trizol and stored at -
154 80°C for further total mRNA extraction.

155 The follicular hemisections were processed from follicles measuring 2-5mm in
156 diameter, which were selected, isolated from the ovaries and dissected free of
157 stromal tissue (Richard and Sirard, 1996). The follicles were sectioned into equal
158 halves with a scalpel blade. The follicular halves were washed in TCM-199 and
159 incubated for 2 h before adding the COCs. The co-culture procedure has been

160 previously conducted in our laboratory (Barreta *et al.*, 2008; Giometti *et al.*, 2005;
161 Stefanello *et al.*, 2006).

162

163 **Statistical analysis**

164 The gene expression results were submitted to analysis of variance (PROC
165 GLM; General Linear Models Procedure). When observed treatment effect, the
166 means were compared using the least squares means (LSMEANS). All continuous
167 variables were tested for normality with the support of the Shapiro-Wilk test and
168 normalized when necessary according to each distribution. The percentages of
169 maturation were analyzed by chi-square test, using PROC CATMOD. The multi-
170 comparison among the different groups was performed by means of contrasts.
171 Analyses were performed using the JMP statistical program (SAS Institute Inc.,
172 Cary, NC) and level of significance was set at 5%. The results of gene expression
173 were represented as mean \pm standard error of the mean for each replication and
174 nuclear maturation are represented in percentages.

175

176 **Results**

177

178 **Validation of the experimental model**

179 To validate our experimental model, we verified the meiotic progression of
180 120 oocytes from follicles of 1-2mm and 3-8mm in diameter (mean of 30 oocytes
181 per treatment). In agreement of previous studies (Arlotto *et al.*, 1996), competent
182 oocyte that underwent to meiotic progression was higher in oocytes from large (3-

183 8mm, 74,3%) than those from small follicles (1-2mm; 32,1%; $p<0.05$). We also
184 assessed the Grb14 mRNA expression in the different compartments of COCs,
185 observing that denuded oocyte and cumulus cells expressed Grb14 mRNA.

186

187 **Profile of Grb14 mRNA expression in COCs during antral follicle development**

188 We assessed Grb14 mRNA expression in COCs from different follicles size
189 related with oocyte developmental competence acquisition (1-3, 4-6, 6-8 and
190 >8mm in diameter). The Grb14 mRNA expression was higher in COCs from
191 follicles of 1-3mm in diameter and decrease significantly and gradually as the
192 follicular size increased. The expression was significantly lower in COCs from
193 largest (>6mm in diameter) than smallest follicles ($p<0.05$; Fig. 1).

194

195 **The estradiol effect on Grb14 mRNA expression**

196 We investigated the role of estradiol in the Grb14 mRNA expression in cattle
197 COCs. Follicular cells and COCs were co-cultured in the presence or absence of E2
198 (500ng/ml) and/or fulvestrant (100 μ M; an estradiol receptor antagonist) for 6 h.
199 The level of Grb14 mRNA expression in the treatments groups (E2, Fulv and/or
200 E2+Fulv) did not differ from the control groups cultured in the absence (positive
201 control, C+) or presence (negative control, C-) of follicular cells ($p>0.05$; Fig.2).

202

203 **Discussion**

204 In this study, we observed that the Grb14 mRNA is expressed in the bovine
205 COCs. To our knowledge, this is the first report of Grb14 mRNA expression in the

206 mammalian COCs. Moreover, we showed that Grb14 is downregulated in COCs
207 as follicular size increases and the expression is not regulated by estradiol. Our
208 results provide evidences of a new candidate gene to be involved in the bovine
209 oocyte maturation, probably controlling factor receptors with tyrosine kinase
210 activity.

211 The COCs were co-cultured with follicular hemisections to delay resumption
212 of meiosis and to continue mRNA transcription (Richard and Sirard, 1996). This
213 culture system has been previously used to study oocyte nuclear maturation
214 (Barreta *et al.*, 2008; Giometti *et al.*, 2005; Stefanello *et al.*, 2006). In this condition,
215 the effects of estradiol and fulvestrant (an estradiol antagonist) were evaluated
216 based on Grb14 mRNA expression in COCs.

217 Cattle oocytes from follicles larger than 2mm in diameter are able to undergo
218 meiotic resumption (Fair *et al.*, 1995). With the validation of our experimental
219 model, we demonstrated that the oocyte competence for meiotic progression
220 increased as the follicle size developed, which has already been described (Arlotto
221 *et al.*, 1996; Lonergan *et al.*, 1994). With this knowledge, the downregulation in
222 Grb14 mRNA expression observed in our study (Fig. 1) suggested that in cattle,
223 oocyte competence for meiosis resumption and metaphase progression is
224 negatively related with Grb14 mRNA expression.

225 In xenopus oocytes, the increase in the Grb14 mRNA expression inhibited the
226 resumption of meiosis and prevented the activation of MAPK and PI3K
227 intracellular signaling pathways. These signaling are essential to promote oocyte
228 GVBD by interacting with phosphorylated tyrosine kinase receptor (Cailliau *et al.*,

229 2003). Taken together, our results and those previously reported suggest that the
230 downregulation in the Grb14 mRNA expression is an important intracellular
231 mechanism to promote the activation of intracellular signaling pathways for
232 acquisition of oocyte developmental competence to undergo GVBD and achieve
233 the M-II. Also, we can infer that Grb14 is involved in the molecular maturation
234 because COCs derived from large follicles (>8mm) had consistently less Grb14
235 mRNA than small follicles (<6mm).

236 The increase level of estradiol in follicular fluid is a key characteristic for
237 dominance during antral follicle development in cattle (Fortune *et al.*, 2004).
238 Moreover, estradiol plays a role in cumulus cell proliferation and expansion
239 (Sugiura *et al.*, 2010). However, we did not observed any estradiol effect on Grb14
240 mRNA expression in COCs during follicle development. In contrast, studies using
241 human breast cancer cells showed that Grb14 protein expression is
242 downregulated by estradiol and upregulated by the antiestrogen ICI 182780
243 (Kairouz *et al.*, 2005). These results lead us to infer two possibilities. Firstly,
244 estradiol might be regulating Grb14 in COCs through a factor not present in our
245 *in vitro* system. Secondly, Grb14 might be regulated by another steroid. Recently,
246 an study demonstrated that dihydrotestosterona downregulated Grb10 (an
247 adaptor protein structurally related to Grb14 and belonging to the Grb7
248 superfamily) mRNA expression in mice skeletal muscle (Svensson *et al.*, 2010).

249 Growth factor signaling by receptor tyrosine kinase induced an increase in
250 oocyte competence and subsequent embryo development (Purohit *et al.*, 2005;
251 Zhang *et al.*, 2010). Considering that Grb14 inhibits factors with tyrosine kinase

252 activity via MAPK and PI3K (Goenaga *et al.*, 2009), the present results are
253 evidencing that Grb14 mRNA expression has to decrease in COCs for oocyte to
254 acquire developmental competence.

255 In conclusion, the Grb14 mRNA expression in COCs was downregulated
256 during follicle development, suggesting a role of this receptor-bound protein in
257 oocyte competence acquisition in cattle. Moreover, we demonstrated that the
258 Grb14 mRNA downregulation is not dependent upon the action of estradiol in
259 cattle COCs.

260

261 **Acknowledgement**

262 We thank the Silva abattoir for providing the bovine ovaries. We also thank
263 CNPq and CAPES for the financial support.

264

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427

428 **List of Figures**

429

430 **Figure 1:** Relative mRNA expression (mean \pm sem) of Grb14 in cumulus-oocyte
431 complexes (COCs) from different follicular sizes (1-3, 4-6, 6-8 and >8mm). The
432 mRNA expression values were relative to the housekeeping gene GAPDH. A total
433 of 480 COCs was evaluated (about 120 COCs/treatment). The experiment was
434 conducted with three replications. Different letters (a, b and c) indicate
435 statistically significant differences between follicles sizes ($p < 0.05$).

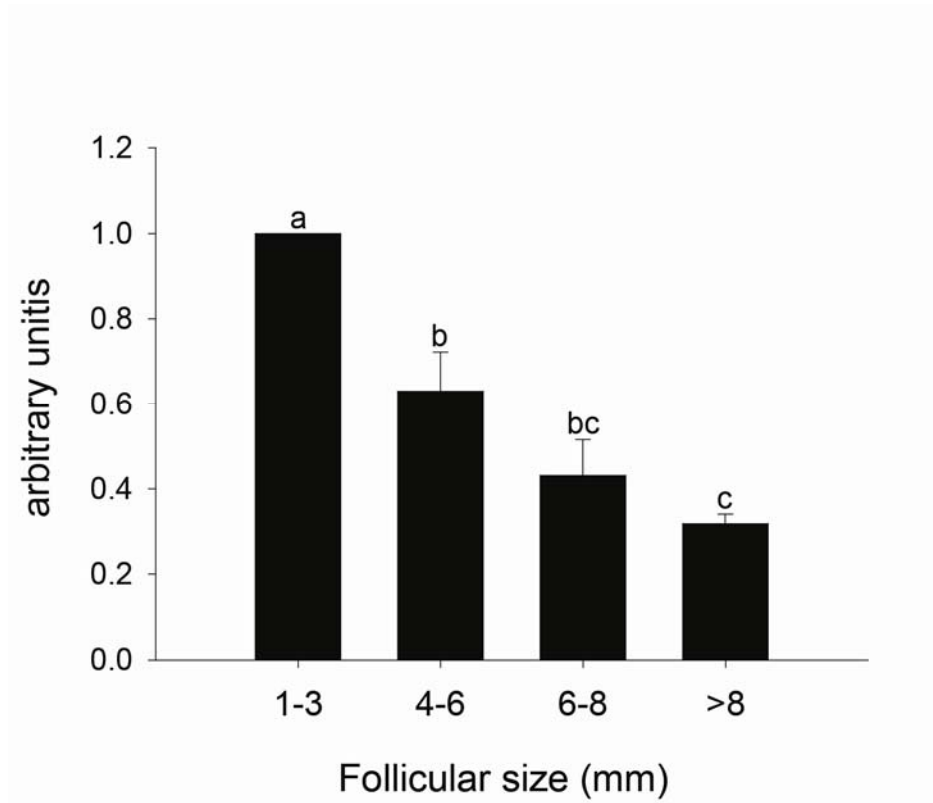
436

437 **Figure 2:** Effect of estradiol and fulvestrant on Grb14 mRNA expression in
438 cumulus-oocyte complexes (COCs). COCs were cultured in the absence (positive
439 control group, C+) or presence (negative control group, C-) of follicular cells for 6
440 h. The COCs in treatment groups were cultured in the presence of follicular cells
441 with 17 β -estradiol (E2; 500ng/ml), fulvestrant (Fulv; 100 μ M) or both (E2+Fulv). A
442 total of 600 COCs (from 3 to 8mm follicles) were allocated in five groups. The
443 experiment was conducted with three replications. There was no statistical
444 difference between groups ($p > 0.05$).

445

446 Figure 1:

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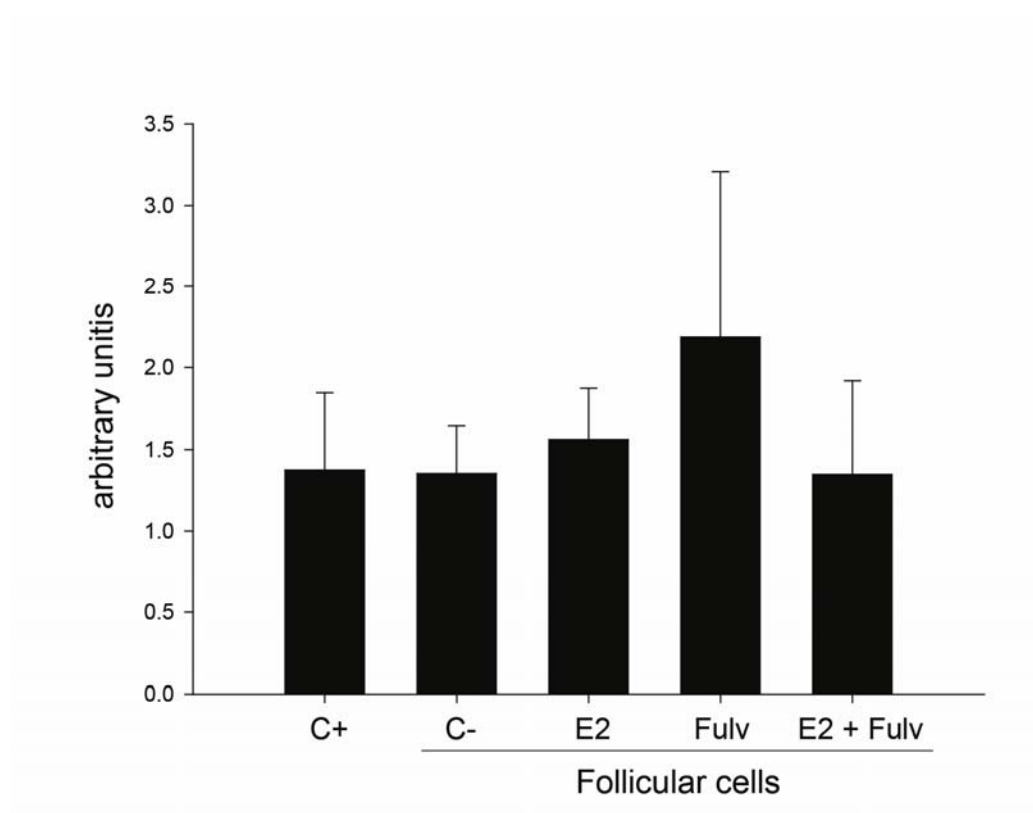


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449 Figure 2:

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ARTIGO 2

TRABALHO A SER ENVIADO PARA PUBLICAÇÃO:

GRB10 CHARACTERIZATION IN CATTLE CUMULUS- OOCYTE COMPLEX FROM DIFFERENT FOLLICULAR SIZES

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Alencar Ludke, Rogério Ferreira, Gabriel Ribas Pereira, Rafael Gianella
Mondadori, João Francisco Coelho de Oliveira, Paulo Bayard Dias
Gonçalves**

Running head: Grb10 characterization in bovine COCs

Grb10 characterization in bovine cumulus oocyte complex from different follicular sizes

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ABSTRACT

The objective of this study was to investigate the mRNA expression and protein localization of Grb10 gene in bovine cumulus-oocyte complex from different follicles size. Firstly, we demonstrated the presence of Grb10 mRNA in oocyte and cumulus cells by qRT-PCR assay. To investigate the expression throughout follicular development and to correlate with maturation rates, COCs from follicles at 1-3, 4-6, 6-8 and >8mm were used to evaluate Grb10 gene expression by qRT-PCR assay and nuclear maturation rates. We observed that more competent oocytes (from follicles at 6-8 and >8mm; $P<0.05$), also had a small Grb10 mRNA expression levels when compared to the oocytes from follicles at 1-3 and 4-6mm ($P<0.05$). We conducted imunofluorescence analysis in the COCs from different follicles size (1-3, 4-6, 6-8 and >8mm) to investigate Grb10 protein localization. Samples were incubated with primary antibodies: Polyclonal rabbit anti-Grb10 (1:100). Primary antibody was detected using goat anti-rabbit IgG antibody conjugated with Alexa Fluor 488 (1:500). Positive fluorescence signal was detected in all analyzed samples but was less evident in COCs from largest follicles. These results show a new gene present in bovine cumulus oocyte complex and provided evidences for involvement of the Grb10 during oocyte molecular maturation.

Key words: Grb10, follicular size, imunofluorescence, oocyte, bovine.

1 INTRODUCTION

2 The growth factor receptor-bound protein 10 (Grb10) is a member of the Grb7 family
3 adaptor molecules that includes Grb10, Grb14 and Grb7 proteins. The Grb7 family of adaptor
4 protein structure is composed by a carboxyl-terminal src-homology 2 (SH2), GM domain and
5 a proline-rich region. Moreover, this central GM domain region has a sequence homology
6 between the Grb7 family proteins that contains a pleckstrin homology (PH) domain, a Ras-
7 association domain and a function region called BPS (between the PH and SH2 domains).
8 The high sequence homology and the conservative molecular architecture between the Grb7
9 family members allow them to participate in the functionality of multiple cellular signal
10 transduction pathways (Han *et al.* 2001; Holt and Siddle 2005).

11 The Grb10 protein is known to have different isoforms that are responsible to bind
12 several trans-membrane tyrosine-kinase receptors, including the insulin receptor (IR), the
13 insulin-like growth factor receptor (IGF-IR) and the epidermal growth factor receptor (EGFR)
14 (Holt and Siddle 2005). Tyrosine kinase receptors residues are autophosphorylated upon
15 binding to growth factors in their extracellular portion. The binding sites of the Grb10 protein
16 to phosphorylated tyrosine kinase receptors are made by the BPS and SH2 domains that
17 inhibit the access of the regulatory subunits of the PI3K and MAPK to the phosphorylated
18 receptor. Throughout this pathway, the activation of the PI3K and MAPK intracellular
19 pathways is inhibited, therefore, important to trigger intracellular modifications in gene
20 expression related to control metabolism and cell proliferation (Lim *et al.* 2004). Interesting,
21 studies in insulin responsive tissue such as skeletal muscle and adipose tissue have shown the
22 presence of Grb10 protein (Ooi *et al.* 1995; Dong *et al.* 1997) and its involvement in tumors
23 responsive to IGF-I, insulin metabolism, diabetes and grown abnormalities (McCann *et al.*
24 2001; Charalambous *et al.* 2003; Lim *et al.* 2004; Goenaga *et al.* 2009; Holt *et al.* 2009).

25 The Grb10 protein is involved in the metabolism of somatic cells by inhibiting the
26 intracellular signaling triggered by tyrosine kinase receptors. Studies performed in HeLa
27 human cell line using small interfering (si)RNA observed that knockdown of Grb10 gene
28 enhances the role of IGF-I mediator in the activation of Akt/PKB (also called protein kinase
29 B), and ERK1/2, with an associated increase in IGF-I mediated DNA synthesis (Dufresne and
30 Smith 2005). Similarly, rat primary adipocytic overexpressing Grb10 inhibits insulin
31 stimulation of MAPK phosphorylation, suggesting that endogenous Grb10 inhibits insulin
32 signaling (Langlais *et al.* 2004).

33 Oocytes undergo several biochemical and morphological changes throughout follicular
34 development. This changes, which begin with the formation of primordial follicles and

35 continue until the time of ovulation, are responsible for the oocyte to undergo suitable
36 fertilization and subsequent embryo development (Brevini Gandolfi and Gandolfi 2001). The
37 oocyte maturation, including cytoplasmatic and molecular events, occurs in a gradual manner
38 and synchronized with the follicular events (Bevers *et al.* 1997). The relationship between
39 follicular size and oocyte competence to undergo complete nuclear maturation and progress to
40 metaphase-II (M-II) *in vitro* has been well described in the bovine (Lonergan *et al.* 1994;
41 Arlotto *et al.* 1996). In addition, the oocytes derived from large follicles are more competent
42 for blastocyst development following *in vitro* fertilization than those derived from smaller
43 follicles (Pavlok *et al.* 1992; Machatkova *et al.* 2004; Lequarre *et al.* 2005).

44 In this study, we investigated the protein Grb10 in cattle cumulus-oocyte complexes.
45 Our hypothesis was based on the evidences that tyrosine kinase receptors have important
46 functions on oocyte molecular maturation during follicular development (Yoshimura *et al.*
47 1996; Nuttinck *et al.* 2004; Jamnongjit *et al.* 2005). Local growth factors such as IGF-I, EGF
48 and fibroblast growth factor 10 (FGF10) added into the maturation medium improves the
49 oocyte maturation, resulting in a high blastocyst rate *in vitro* (Purohit *et al.* 2005; Wang *et al.*
50 2009; Zhang *et al.* 2010). In addition, events during the bovine oocyte maturation are
51 regulated by intracellular pathways to promote cell proliferation and metabolism such as
52 mitogen-activated protein kinase (MAPK) also called “extracellular signal-regulated kinase”
53 (ERK) and maturation promoting factor (MPF). The increase in the activities of MPF and
54 MAPK in cattle oocytes is necessary for the germinal vesicle breakdown (GVBD) and
55 metaphase progression (Nurse 1990; Fissore *et al.* 1996).

56 The presence of the Grb10 mRNA and protein expression has not yet been
57 investigated in mammalian oocyte, despite the importance of growth factors that bind to
58 receptors with tyrosine kinase activity during oocyte molecular maturation. Thus, the
59 objectives of this study were to evaluate the Grb10 mRNA and protein expression in COCs
60 from follicles at different growing stages relating this to different oocyte competence for
61 meiotic progression.

62

63 **MATERIALS AND METHODS**

64

65 ***Cumulus-oocyte complexes processing for real-time RT-PCR***

66 Bovine ovaries were obtained from abattoir and transported cooled (5°C) in a thermal
67 container to the laboratory (for better preservation of RNA integrity), containing saline
68 solution (0.9% NaCl), 100 IU/ml penicillin (Sigma Chemical Company, St. Louis, MO, USA)

69 and 50 µg/ml streptomycin (Sigma). In the laboratory, follicles (with diameters of 1-3, 4-6, 6-
70 8 and >8mm) were aspirated with a vacuum pump (vacuum rate of 20 ml/minute). The COCs
71 were recovered and selected under a stereomicroscope according to Leibfried and First
72 (1979). Cumulus-oocyte complexes, grade 1 and 2, were washed three times in PBS and
73 placed into a microtube containing 200µl trizol for further extraction of total RNA.

74

75 ***Assessment of oocyte nuclear maturation***

76 The COCs from different follicular size (1-3, 4-6, 6-8 and >8mm) were cultured in
77 maturation medium for 20 h to evaluate meiotic competence and relate to the expression
78 levels and presence of Grb10 mRNA. Briefly, after recovery, the COC's were transferred into
79 four-well Petri dish (Nunc Intermed., Roskilde, Denmark) containing 400µl tissue culture
80 medium-199 (TCM-199) with Earle's salts, L-glutamine, Hepes and sodium bicarbonate
81 (Gibco Labs, Grand Island, NY, USA), supplemented with 100UI/ml penicillin, 50µg/ml
82 streptomycin, 5µg/ml LH (Lutropin-V®, Bioniche, Ontario/CA), 0.5g/ml FSH (Folltropin®-
83 V, Bioniche, Ontario/CA), 0.2mM sodium pyruvate and 0.4% BSA. The COCs were cultured
84 at 39°C in an atmosphere of 5% CO₂ in air with saturated humidity as described by Stefanelli
85 et al. (2006). Then, the cumulus cells were removed by vortexing and oocytes were fixed in
86 4% paraformaldehyde during 15 min and transferred to 0.5% Triton-X-100. After, oocytes
87 were stained with 10µg/ml bisbenzimidazole (Hoechst 33342) and analyzed under a fluorescence
88 microscope. Oocyte maturation status was classified as immature [germinal vesicle (GV),
89 GVBD, metaphase-I (M-I)] and mature [anaphase-I (AI), telophase-I (TI), and metaphase II
90 (M-II)].

91

92 ***Nucleic acid extraction and real-time RT-PCR***

93 Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) protocol according
94 to the manufacturer's instructions and was quantified by absorbance at 260 nm using a
95 spectrophotometer NanoDrop (Thermo Fischer Scientific Inc., Waltham, MA). The total RNA
96 integrity was verified electrophoretically by ethidium bromide staining and purity was
97 determined by absorption rate relationship of OD₂₆₀/OD₂₈₀. Only total RNA samples
98 containing values >1.7 were used in this experiment.

99 Total RNA (1 mg) was first treated with 0.2U DNase (Promega, Madison, WI) at 37°C
100 for 30 min to digest any contaminating DNA, followed by heating to 65°C for 3 min. The
101 RNA was reverse transcribed (RT) in the presence of 1µM oligo (dT) primer, 4U Omniscript
102 RTase (Omniscript RT Kit; Qiagen), 0.5µM dideoxynucleotide triphosphate (dNTP) mix, and

103 10U RNase inhibitor (Invitrogen) in a volume of 20 μ L at 37°C for 1 h. The reaction was
104 terminated by incubation at 93°C for 5 min.

105 The relative gene expression was assessed by real-time RT-PCR (RT-PCR) using the
106 StepOnePlus™ RT-PCR system (Applied Biosystems, Foster City, CA). All samples were
107 analyzed in duplicate and each sample contained 12.5 μ l of SYBR Green PCR Master Mix
108 (Applied Biosystems), 8.5 μ l of H₂O, 1 μ l of forward primer (200nM), 1 μ l of reverse primer
109 (200nM) and 2 μ l of cDNA. The reaction was carried out as following: 50°C for 2 min, 95°C
110 for 10 min, 40 cycles at 95°C for 15 sec and 60°C for 1 min. Melting-curve analyses were
111 performed to verify product identity. The variability in the amount of mRNA was corrected
112 by amplification of GAPDH housekeeping gene, and relative expression was performed as
113 recommended by Pfaffl (2001). The primers for Grb10 (F- GGAGATTCTGGCAGACATGA
114 and R- TAATCCAGGTGTGGGTGAT) and GAPDH (F- GATTGTCAGCAATGCCTCCT
115 and R- GGTCATAAGTCCCTCCACGA) were designed in the Primer Express software v 3.3
116 (Applied Biosystems) and synthesized by Invitrogen.

117

118 *Immunofluorescence assessment*

119 Bovine follicles at different size 1-3, 4-6, 6-8 and >8mm were isolated from the
120 ovaries and fixed into a 4% paraformaldehyde solution at 4°C for 12h and paraffin embedded
121 for further evaluation of Grb10 protein. Human liver sample (size about 2mm) was used as
122 positive control of Grb10 gene expression (Holt and Siddle 2005). Histological sections with
123 3 μ m were prepared to perform immunofluorescence analysis. Slides were deparaffinized
124 using Xylene for 15 min, rehydrated through a graded alcohol series (one times for five min in
125 each 100%, 90%, 80%, 70% and 50% dilution), and rinsed for 15 min in distilled water.
126 Endogenous peroxidase activity was then blocked for 20 min in hydrogen peroxide 0.3% and
127 washed three times in PBS1X for 5 min. After washing, the slides were carefully blotted
128 using a PAP pen (Vector Laboratory, Burlingame, CA) around the tissue. A blocking solution
129 (PBS1X with 3% of Bovine Serum Albumin and 0.2% Twen-20) was used to block non-
130 specific sites during 2 h at room temperature in a humidify chamber. After washed three times
131 in PBS1X during 5 min, the same blocking solution was used to incubate with the primary
132 Grb10 antibody (dilution 1:100; Santa Cruz Biotechnology) in a humidified chamber
133 overnight at 5°C. After this incubation, samples were washed three times in a PBS1X
134 containing 0.2% Tween-20 for 5 min before being incubated for 1 h at room temperature to a
135 goat anti-rabbit IgG antibody conjugated with AlexaFluor 488 (dilution 1:500; Invitrogen).
136 Then, slides were washed in three times in a PBS1X containing 0.2% Tween-20 for 5 min.

137 Finally, to enable nuclear staining visualization, samples were incubated with 300nM of 4',6-
138 diamidino-2-phenylindole (DAPI; Invitrogen) in PBS1X for 5 min at room temperature.
139 Then, slides were mounted with a space between the coverslip, filled with 50µl drop of
140 Aqueous Mounting Medium (Fluoromount) and sealed with nail polish. Laser-scanning
141 confocal microscopy was performed using a Confocal Microscope Spectral FV1000
142 (Olympus). Laser scanning microscope was equipped with two lasers for the simultaneous
143 excitation of Alexa Fluor 488 fluorescent for Grb10, and DAPI for DNA, with fluorescence
144 excitation and emission of 495/518 and 358/461nm, respectively. Image software FV-Viewer
145 (Olympus) was used to obtain sample images.

146

147 *Statistical Analysis*

148 The results of gene expression were compared by analysis of variance (PROC GLM;
149 General Linear Models Procedure). When observed an effect of treatment, the means between
150 the different groups were compared using the multi-comparison of means test least squares
151 means (LSMEANS). All continuous variables were tested for normality with the support of
152 the Shapiro-Wilk test and normalized when necessary according to each distribution. The
153 analysis of the percentage of maturation in different groups was performed by chi-square test,
154 using PROC CATMOD. The multi-comparison among the different groups was performed by
155 means of contrasts. Analyses were performed using the statistical program SAS and adopted
156 the significance level of 5%. The results of gene expression are represented as mean \pm
157 standard error of the mean for each replication and nuclear maturation are represented in
158 percentage form.

159

160 **RESULTS**

161

162 *Grb10 mRNA expression in COCs from follicles at different stages of development*

163 Oocytes from follicles of 1-3, 4-6, 6-8 and >8mm in diameter, grade 1 and 2, were
164 assessed for nuclear maturation in order to characterize the model used to study Grb10 gene
165 expression. Confirming previous studies (Yang *et al.* 1998; Marchal *et al.* 2002), the oocytes
166 from follicles with 1-3 or 4-6mm in diameter had lower ($P < 0.05$) maturation rates than those
167 from 6-8 or >8mm in diameter (Figure 1A). Initially, Grb10 mRNA expression was evaluated
168 in isolated oocytes and cumulus cells by RT-PCR assay in order to validate the model.
169 Considering that Grb10 mRNA was expressed in oocyte and cumulus cells, cumulus-oocyte
170 complexes from different follicles size were used in subsequent experiments. The results

171 showed that Grb10 mRNA expression were higher ($P < 0.05$) in COCs from follicles with 1-3
172 and 4-6mm in diameter than in COCs from follicles with 6-8 and >8mm in diameter (Figure
173 1B). The Grb10 mRNA expression in COCs was inversely related to oocyte competence for
174 meiotic progression to mature stage.

175

176 ***Localization of Grb10 protein in COCs***

177 Immunofluorescence confocal microscopy of COCs from follicles at different stages
178 of development revealed positive fluorescence signal for Grb10 protein in oocyte and
179 cumulus cells of all COCs analyzed (Figure 2). Interestingly, the positive fluorescence
180 signaling was less evident in cumulus cells from follicles of 6-8 and >8mm in diameter than
181 those cells of ≤ 6 mm follicles. The negative control samples cells were not stained in the
182 absence of primary antibody. Human liver was used as positive control (Figure 2).

183

184 **DISCUSSION**

185 In this study, we demonstrated the presence of Grb10 mRNA and protein expression in
186 bovine cumulus-oocyte complexes. To our knowledge, this is the first study to show cell-
187 specific localization of Grb10 protein and mRNA expression in mammalian COCs. Moreover,
188 the expression of Grb10 mRNA in COCs seems to be inversely correlated with oocyte
189 competence. The expression of Grb10 protein is clearly reduced in cumulus cells of >6mm
190 follicle when compared with those of <6mm follicle. On the other hand, oocyte competence to
191 achieve embryo development increases proportionally to follicular size (Lonergan *et al.*
192 1994). Therefore, these results suggest that Grb10 is involved in acquisition of oocyte
193 competence throughout antral follicle growth.

194 During follicular development, COCs undergo extensive proliferation and
195 differentiation (Armstrong *et al.* 1996). Grb10 seems to be involved in this two process
196 through interaction with tyrosine kinase receptors. In mouse embryo fibroblast cell lines, the
197 IGF-I-mediated mitogenesis is inhibited by Grb10 (Morrione *et al.* 1997). In addition, Grb10
198 over-expression inhibits insulin-stimulated glycogen synthesis in rat hepatocytes (Mounier *et*
199 *al.* 2001). Taken together, our results and those previously reported suggest that in cattle
200 COCs, Grb10 is involved in proliferative events controlling growth factors receptors with
201 tyrosine kinase activity.

202 To validate our experimental model, we showed that oocytes from largest follicles (6-
203 8 an >8mm in diameter) had a higher maturation rate compared to oocytes from small
204 follicles (1-3 and 4-6mm). Our results demonstrated that Grb10 mRNA expression is lower in

205 COCs derived from larger diameter follicles (>6mm), which contain more competent oocytes.
206 In the same way, a similar work showed that bovine oocytes derived from follicles at >6mm
207 in diameter have a significantly higher rates of development to cleavage or blastocyst stages
208 than oocytes from small follicles (Caixeta *et al.* 2009). This finding indicates a particular
209 relationship between follicular size, Grb10 mRNA expression and oocyte competence
210 acquisition, suggesting that the decreased of Grb10 mRNA expression in COCs from largest
211 follicles can be an important intracellular signaling for the oocyte competence acquisition for
212 meiotic progression and embryonic development.

213 Growth factor receptor-bound protein 10 plays a pivotal role in multiple intracellular
214 transduction pathways after binding in activated tyrosine kinase receptors (Lucas-Fernandez
215 *et al.* 2008). For the success of maturation process, changes in transcriptional activity are
216 needed for oocyte undergo fertilization and support early embryonic development. In this
217 context, some genes increase their expression levels in mammalian COCs during follicular
218 development while others decrease to obtain optimal oocyte molecular maturation
219 (Hernandez-Gonzalez *et al.* 2006; Mamo *et al.* 2011). These previous data in association with
220 the decrease in the Grb10 mRNA and protein expression in COCs from largest follicles
221 (>6mm) observed in our results, suggest that Grb10, by binding with activated tyrosine kinase
222 receptors, modulate signal transduction pathways of the key genes involved in oocyte
223 molecular maturation during antral follicular development in cattle.

224 Mammalian cumulus cells have important functions during oocyte maturation process
225 such as: slow steroidogenesis activity and hyaluronic acid production for cumulus expansion
226 (Eppig 2001). For cumulus cells play these mentioned functions, some genes needs to be
227 down-regulated such as Nrip1 (Sugiura *et al.* 2010). Recent studies have shown that Nrip1
228 plays an important role in controlling lipid and glucose metabolism. Mice with disruption of
229 Nrip1 are lean and resistant to high-fat diet with improve insulin sensitivity (Rosell *et al.*
230 2010). In agreement, a study using knockout mice with a disruption of the Grb10 gene was
231 found to improve insulin sensitivity and to reduce adiposity (Smith *et al.* 2007). An
232 association of Nrip1 and Grb10 has not been described.

233 Growth factor receptor-bound protein 10 seems to be related as imprinted gene growth
234 suppressor in mice and rat. Previous studies showed that the disruption of Grb10 maternal
235 allele gene caused an overgrowth in mice at birth (Charalambous *et al.* 2003) and in adult rat
236 (Wang *et al.* 2007). So, this failure in embryonic development may be due to lack of
237 accumulated mRNA and protein for Grb10 gene in female gamete. Levels of RNAs and
238 proteins synthesized and stored by the oocyte are very important to sustain embryonic

239 development (Lonergan *et al.* 1998). Taken together, this previous data and the
240 characterization of Grb10 mRNA and protein in bovine COCs showed in our study, suggest
241 an involvement of this gene in bovine oocytes throughout follicular development until
242 embryonic phase.

243 In conclusion, we demonstrated the presence of Grb10 mRNA expression and protein
244 localization in bovine cumulus-oocyte complexes. The presence and the differential
245 expression of Grb10 mRNA in COCs from follicles at different developmental stages suggest
246 the involvement of Grb10 in the oocyte molecular maturation.

247

248 **ACKNOWLEDGEMENTS**

249 This work was supported by CNPq and CAPES. We would like to thanks Henrique
250 Beck Biehl from the Center of Electron Microscopy at the Federal University of Rio Grande
251 do Sul (UFRGS) for assistance with confocal microscopy. Dra. Cristine Kolling Konopka
252 from University Hospital of Santa Maria for human liver sample kindly provided and Silva
253 abattoir for providing the bovine ovaries utilized in the study.

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427 List of Figures

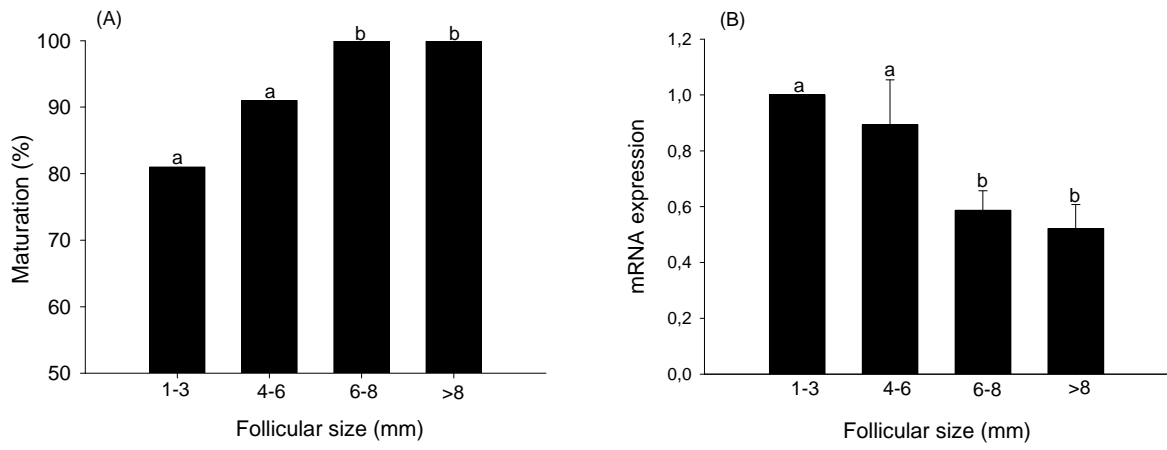
428

429 Figure 1: (A) Nuclear maturation rate of oocytes from follicles at different stages of
430 development. The oocytes in AI, TI and MII were classified as matured. A total of 120
431 oocytes (~ 30 oocytes/treatment; mean of 15 oocytes/treatment/replication). Different letters
432 represent significant differences between groups ($P<0.05$). This experiment was performed in
433 duplicate. (B) Grb10 mRNA expression relatively to the housekeeping gene GAPDH in
434 COCs from follicles at different stages of development. A total of 480 COCs (~120
435 COCs/treatment; mean of 40 COCs/treatment/replication). Different letters (a and b) above
436 error bars indicate statistically differences between follicle sizes ($P<0.05$). This experiment
437 was performed in triplicate

438

439 Figure 2: Immunofluorescence localization of Grb10 protein in COCs derived from
440 follicles at different size (1-3, 4-6, 6-8 and >8mm). Positive fluorescence signal (green) for
441 Grb10 was detected in the oocyte and cumulus cells of all treatment groups. Control
442 immunofluorescence was performed by omitting the primary antibody. DAPI, 4',6-Diamidino-
443 2-phenylindole counter staining (blue); Oo, oocyte; ZP, zona pellucida; Cc, cumulus cells.
444 Human liver was used as a positive control. Magnification 40x. Bars represent 50 μ m.

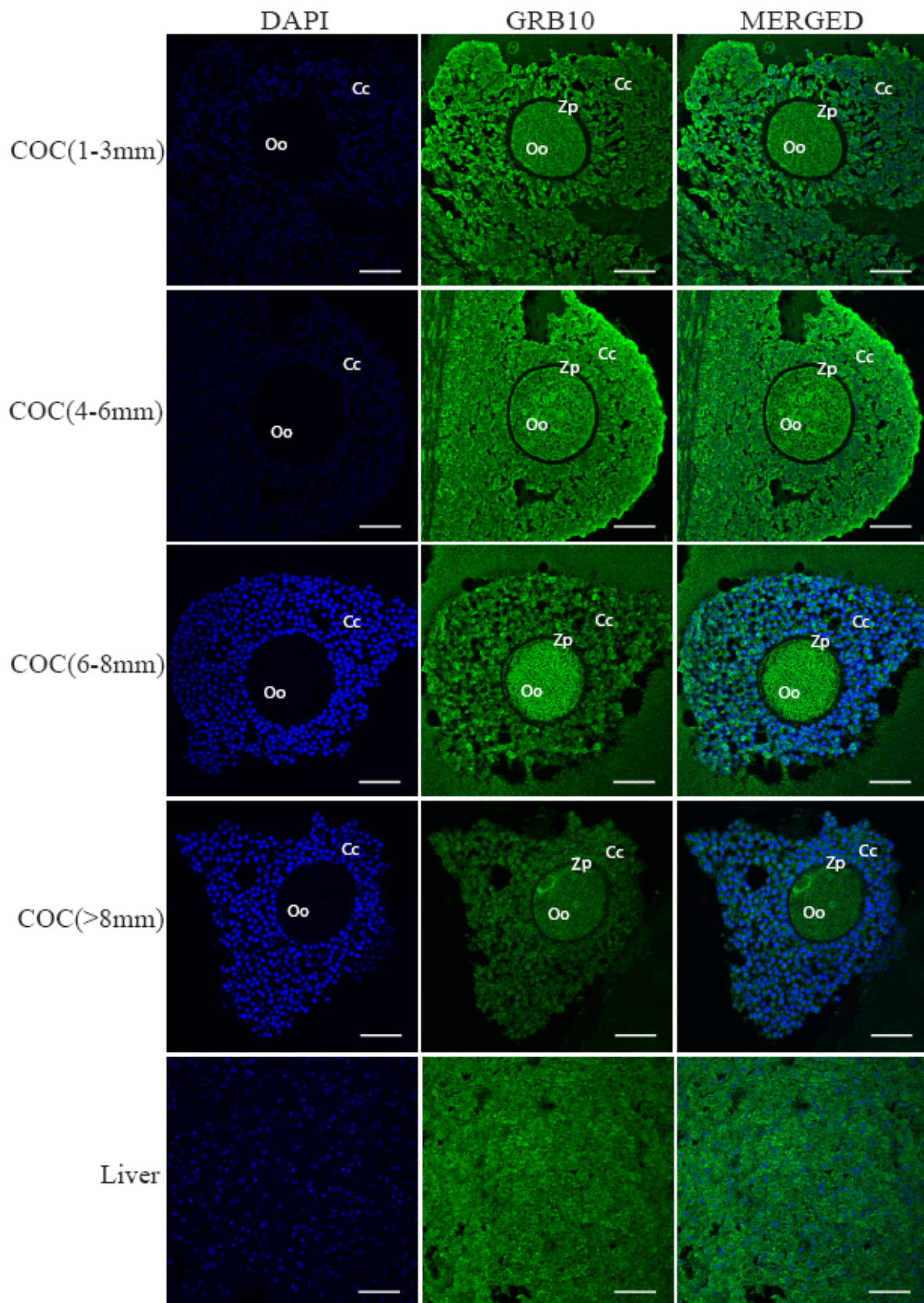
445 Figure 1
446



447

448

Figure 2



449

450

RESULTADOS COMPLEMENTARES

Expressão de RNAm para Grb10 em CCOs após cultivo em meio suplementado com estradiol e/ou fulvestrant

O objetivo deste experimento foi avaliar se o estradiol é capaz de regular a expressão de RNAm para Grb10. Foi usado um sistema de cultivo *in vitro* de CCOs durante 6h na presença de células foliculares (2 metades de folículos de 2-5mm para cada 50µl de meio). As células foliculares foram utilizadas com o intuito de manter os oócitos no estágio de vesícula germinativa (VG) para que a atividade transcricional não cesse e possa ser avaliado o efeito do tratamento na expressão de RNAm para Grb10. Foram realizadas três replicações nesse experimento com um total de 600 CCOs oriundos de folículos de 3-8mm. Foram utilizados 4 grupos de CCOs (40 CCOs por tratamento) cultivados em gotas de 200µl de meio TCM-199 (Gibco Labs, Grand Island, NY, USA) suplementado com 17β-estradiol (500ng/ml), fulvestrant (100µM) ou ambos em associação. Após 6h os grupos de CCOs foram acondicionados separadamente em eppendorf contendo 200µl de trizol e congeladas a -80°C para extração de RNAm.

A expressão relativa de Grb10 foi realizada por qRT-PCR e a variabilidade na quantidade de RNAm corrigida pela amplificação do gene constitutivo GAPDH. As sequências dos primers para Grb10 (iniciador sense: GGAGATTCTGGCAGACATGA; iniciador anti-sense: TAATCCCAGGTGTGGGTGAT) e GAPDH (iniciador sense: GATTGTCAGCAATGCCTCCT; iniciador anti-sense: GGTCATAAGTCCCTCCACGA) foram desenhados no programa Primer Express program v 3.3 (Applied Biosystems) e sintetizados pela Invitrogen. Os cálculos para expressão relativa de Grb10, bem como a análise estatística, foram realizados como descrito anteriormente no capítulo 1 para o Grb14.

O estradiol utilizado no meio não foi capaz de inibir a expressão de Grb10, da mesma forma que o fulvestrant não foi capaz de aumentar a expressão do Grb10 conforme esperado. Não houve diferença estatística entre os tratamentos.

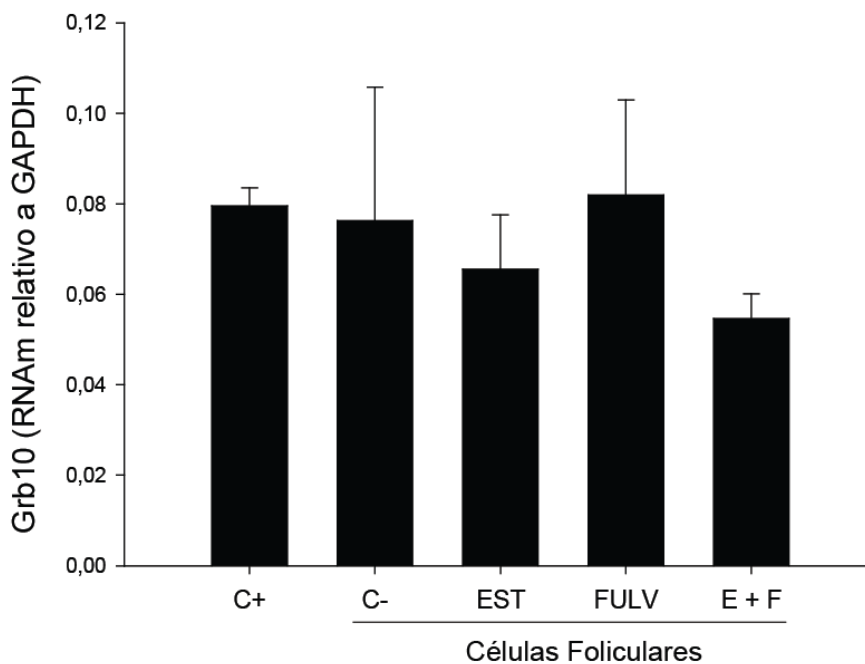


Figura 4 – Expressão relativa de RNAm (média \pm erro padrão da média) para Grb10 em CCOs bovino após 6h de maturação em meio suplementado com estradiol e/ou fulvestrant na presença de células foliculares. C+ (Controle Positivo): Ausência de células foliculares; C- (Controle negativo): Presença de células foliculares no meio de maturação; EST: Meio de maturação suplementado com 17 β -estradiol (500ng/ml) na presença de células foliculares; FULV: Meio de maturação suplementado com fulvestrant (100 μ M) na presença de células foliculares; E + F: Meio de maturação suplementado com 17 β -estradiol (500ng/ml) e fulvestrant (100 μ M) na presença de células foliculares. Foram realizadas três replicações ($P > 0,05$).

DISCUSSÃO

O processo de maturação oocitária *in vivo* ocorre paralelo ao desenvolvimento folicular com os eventos foliculares e oocitários ocorrendo concomitantemente (BEVERS et al., 1997). Dessa forma oócitos oriundos de folículos em estádios adiantados de desenvolvimento apresentam uma maior competência ao desenvolvimento embrionário *in vitro* quando comparados aos oriundos de folículos em estádios precoce de desenvolvimento (YANG et al., 1998a; LEQUARRE et al., 2005).

Durante o processo de maturação oocitária ao longo do desenvolvimento folicular, diversos genes atuam positiva ou negativamente na aquisição de competência oocitária. Alguns trabalhos que utilizam expressão gênica ou microarranjos como abordagem para o estudo de genes envolvidos na maturação oocitária, mostram uma mudança no perfil de expressão de vários genes ao comparar oócitos imaturos com oócitos maturados *in vitro* (MAMO et al., 2011). Além disso, um estudo em camundongos demonstrou uma variação no perfil de expressão de diversos genes em CCOs ao comparar momentos antes ou após a ovulação (HERNANDEZ-GONZALEZ et al., 2006).

Uma vez que as proteínas Grb10 e Grb14 regulam rotas intracelulares ativadas por fatores de crescimento que se ligam em receptores do tipo tirosina quinase (HOLT & SIDDLE, 2005), associado ao envolvimento desses fatores no processo de maturação oocitária e foliculogênese (RIVERA & FORTUNE, 2003; LI et al., 2004; PUROHIT et al., 2005; ZHANG et al., 2010) nosso grupo começou a caracterizar a expressão de RNAm para Grb10 e Grb14 e a localização da proteína Grb10 em CCOs de bovinos. Além disso, devido a importância do estradiol em regular a expressão da proteína Grb14 em células mamárias cancerígenas de mulheres (KAIROUZ et al., 2005), estabelecemos a hipótese de que ocorre variação nos níveis de expressão de RNAm para Grb10 e Grb14 em CCOs oriundos de folículos em diferentes fases de desenvolvimento e estipulamos o estradiol como potente regulador da expressão de RNAm para as proteínas em estudo.

O presente estudo foi baseado em várias metodologias para caracterizar as proteínas Grb10 e Grb14 e demonstrar um possível efeito hormonal na regulação da expressão de RNAm: 1) recuperação de oócitos e células do cumulus de um pool de CCOs oriundos de folículos de 3-8mm de diâmetro e avaliação da expressão gênica através da técnica de RT-PCR em tempo real. 2) Avaliação da taxa de maturação nuclear de oócitos oriundos de folículos de diferentes tamanhos. 3) recuperação de CCOs oriundos de folículos de diferentes

tamanhos (1-3, 4-6, 6-8 e >8mm) e avaliação da expressão de RNAm pela técnica de RT-PCR em tempo real. 4) Localização da proteína Grb10 em CCOs oriundos de folículos de diferentes tamanhos (1-3, 4-6, 6-8 e >8mm) pela técnica de imunofluorescência. 5) Cultivo de CCOs na presença de células foliculares em meio suplementado com 17β estradiol e/ou fulvestrant (antagonista do estradiol) e posterior análise da expressão de RNAm com a utilização da técnica de RT-PCR em tempo real. Com essas metodologias nossos principais achados foram: 1) Ocorre expressão de RNAm para Grb10 e Grb14 tanto no oócito quanto nas células do cumulus, e essa expressão é mais evidente no oócito que nas células do cumulus. 2) A expressão de RNAm para Grb10 em CCOs oriundos de folículos de 1-3 e 4-6mm é significativamente maior que em CCOs oriundos de folículos de 6-8 e >8mm. Similarmente, a expressão de RNAm para Grb14 é maior em CCOs oriundos de folículos de 1-3mm e diminui significativamente conforme o aumento do tamanho do folículo de origem. 3) A proteína Grb10 foi localizada tanto no oócito quanto nas células do cumulus de CCOs oriundos de folículos de diferentes tamanhos (1-3, 4-6, 6-8 e >8mm), porém a localização foi mais evidente no oócito que nas células do cumulus independente do tamanho do folículo de origem. 4) Não ocorre regulação na expressão de Grb10 e Grb14 em CCOs cultivados em meio suplementado com estradiol e/ou fulvestrant.

Estudos prévios demonstraram que o Grb14 está envolvido no reinício da meiose de oócitos de xenopus através da interação com receptores do tipo tirosina quinase e modulação das rotas intracelulares MAPK e PI3K (CAILLIAU et al., 2003; GOENAGA et al., 2009b; BROWAEYS-POLY et al., 2010). Esses dados colaboram com a nossa hipótese de um possível envolvimento das proteínas Grb10 e/ou Grb14 durante a maturação de oócitos bovinos, uma vez que, os nossos resultados demonstram que a expressão diferencial de RNAm para as proteínas em estudo em CCOs oriundos de folículos de diferentes tamanhos parece estar relacionado com o aumento nas taxas de competência ao reinício da meiose e progressão até metáfase II.

A expressão de RNAm e localização da proteína Grb10 em CCOs oriundos de folículos em diferentes estádios de desenvolvimento além de sugerir um envolvimento no processo de maturação, também sugere uma participação nos estádios iniciais de desenvolvimento embrionário uma vez que, segundo CHARALAMBOUS (2003), a prole de camundongos nulos para o gene Grb10 apresentam crescimento acelerado com mortes após o nascimento.

Estudos demonstram a participação de hormônios esteróides na regulação da expressão das proteínas Grb10 ou Grb14 (KAIROUZ et al., 2005; SVENSSON et al., 2010).

Com base nos dados desses estudos prévios, realizamos um experimento cujo objetivo foi avaliar o efeito do estradiol (esteróide fundamental para o desenvolvimento folicular e estabelecimento da dominância) na expressão de RNAm para Grb10 e Grb14 em CCOs de bovinos. No modelo experimental *in vitro* que utilizamos, o estradiol suplementado no meio de maturação não alterou significativamente os níveis de expressão de RNAm para as proteínas em estudo. Portanto, não descartamos a hipótese de que há uma regulação por hormônio esteróide, porém, em CCOs de bovinos, sugerimos que o estradiol não é o esteróide responsável para tal regulação. Dessa forma, novos estudos são necessários para melhor compreendermos um envolvimento das proteínas Grb10 e/ou Grb14 ao longo do processo de maturação de oócitos bovinos.

Este foi um estudo pioneiro na identificação dos genes Grb10 e Grb14 em CCOs de mamíferos. Os dados aqui apresentados contribuem com estudos futuros envolvendo a caracterização funcional dessas proteínas. Podem ainda servir como ferramenta para um melhor entendimento dos mecanismos intracelulares responsáveis pela aquisição de competência oocitária à progressão meiótica e subsequente desenvolvimento embrionário.

CONCLUSÃO

Os resultados do presente estudo permitem concluir que ocorre expressão de RNAm para Grb10 e Grb14 em CCOs de bovinos. Da mesma forma, a proteína Grb10 está presente em CCOs, tanto no oócito quanto nas células do cumulus, oriundos de folículos de diferentes tamanhos. A expressão diferencial do Grb10 e do Grb14 em CCOs de folículos de diferentes tamanhos são evidências da participação dessas proteínas no processo de aquisição de competência oocitária ao longo do desenvolvimento folicular. No entanto, nas condições testadas, o estradiol, fundamental para o desenvolvimento folicular, parece não estar envolvido na regulação da expressão de RNAm para Grb10 e Grb14.

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