Spring 2015

The influence of extracellular-regulated kinases (ERKs) on granulosa cell fate in the bovine follicle

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The Influence of Extracellular-Regulated Kinases (ERKs) on Granulosa Cell Fate in the Bovine Follicle

Honors Senior Thesis
May 2015

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Abstract

Each year, infertility in cows costs the dairy industry over $400 million. This project is focused on the onset of follicular atresia in cows; the process by which the egg-bearing follicles die prematurely and can lead to infertility. The cellular mechanisms that influence programmed cell death, or apoptosis, of bovine granulosa cells (bGCs) within follicles are being investigated. In general, the extra-cellular regulated kinases (ERKs) are intracellular signaling proteins believed to influence the proliferation, survival, and differentiation of cells. In the context of follicular atresia, however, the relationship between the onset of apoptosis of bGCs and the effects of ERKs is relatively unknown. In the present study we are utilizing a constitutively active mitogen activated protein kinase (MEK1) to upregulate ERKs in bGCs, and then determine the effect on bGC sensitivity to apoptosis. Cultured bGCs were genetically modified via transduction using a constitutively active MEK1 adenovirus. Currently we are verifying the efficiency of viral transduction, overexpression of MEK-1, and downstream activation of ERK by flow cytometry and immunoblotting. Preliminary results indicate a lack of MEK1 up-regulation in transduced bGCs. Subsequent experiments will focus on optimizing adenovirus transfection in order to test the hypothesis that upregulated ERKs prevent cytokine-induced apoptosis of bGC.

1. Introduction

Infertility and the role of ERKs

A major cause of infertility in the dairy industry is follicular atresia, the process by which the egg-bearing follicle dies prematurely. During a typical ovarian cycle, cohorts of follicles are recruited and grow in successive waves, ultimately leading to the maturation of a select few follicles. In this process a single follicle is selected and becomes dominant, whereas the other remaining follicles of the cohort become subordinate and eventually undergo follicular atresia. The dominant follicle then either ovulates or is eliminated by follicular atresia, similar to the subordinate follicles (Sirois, 1988; Ginther, 1989). The cellular mechanisms that ultimately dictate the growth or atresia of follicles, however, are relatively unknown.

Follicular atresia occurs in part by an upregulation of cytokine receptors on the cell surface, such as Fas ligand, tumor necrosis factor (TNF), and TNF receptor-associated inducing ligand (TRAIL). This receptor upregulation is associated with the elimination of subordinate follicles in the bovine ovary (Kim, 1998). Thus the cell-specificity of apoptosis within the granulosa cell layer is a critical part of follicular atresia. It has been proposed that the vulnerable cells lack or have lost "pro-survival" signaling mechanisms that resistant cells retain or have gained. The mitogen activated protein kinase pathway (MAPK) is considered a growth and pro-survival pathway, and therefore is central to this study.

We propose that one of the cellular mechanisms that influences granulosa cells, and thus follicular fate, are the extracellular-regulated kinases (ERKs) of the MAPK pathway. Their activation and expression influence proliferation, survival, and differentiation of

granulosa cells of dominant verse subordinate follicles. The ERKs are upregulated in future dominant follicles (Ryan, 2007). Furthermore, it is also at this point in folliculogenesis that granulosa cells either become vulnerable or resistant to apoptosis. The relationship between the expression of ERKs and the incidence of apoptosis, however, is relatively unknown. Since MAPK signaling is known as a growth and pro-survival pathway, we propose that ERK signaling provides resistance to apoptosis of bovine granulosa cells.

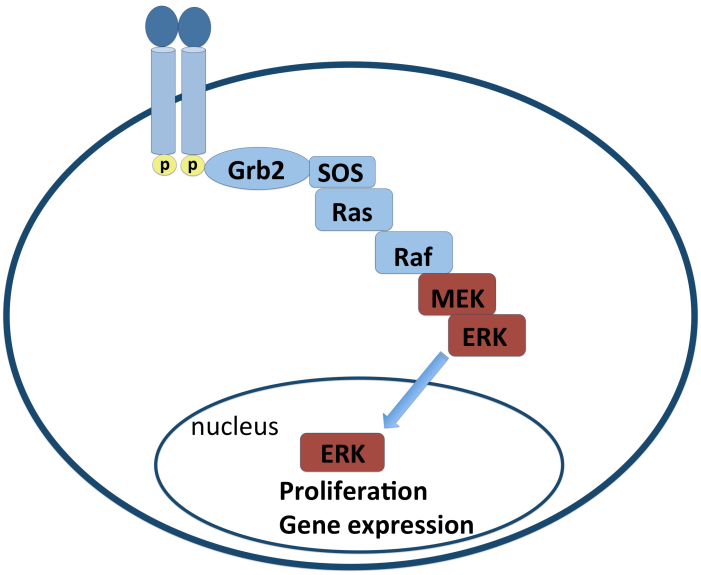

Figure 1: Diagram of the Mitogen Activated Protein Kinase (MAPK) pathway in bGCs.

Adenovirus as a vehicle for MEK1 transduction

In order to test our hypothesis that ERK signaling provides resistance to apoptosis in bovine granulosa cells, we attempted to upregulate the upstream protein MAPK/ERK kinase (MEK1), to phosphorylate ERK, which is next in line in the activation cascade. To do this, we used an adenovirus vector containing a constitutively active MEK1 gene. Transduction refers to virus-mediated DNA transfer into cells. Replication-deficient

recombinant adenoviruses are widely used in research for this purpose. These modified viruses lack genes E1 and E3 for replication, but retain the ability to infect target cells.

Attachment of adenovirus to target cells is mediated by high-affinity binding to the Coxsackie-Adenovirus Receptor (Philipson, 2004). Internalization of the adenovirus occurs via endocytosis after interaction with αv-integrins. The adenovirus reaches the nucleus by transport provided by microtubules and injects its DNA into it. Once in the nucleus, the viral DNA remains epichromosomal (Riedl, 2011). Theoretically, the DNA would then be transcribed into mRNA and translated into MEK1 protein.

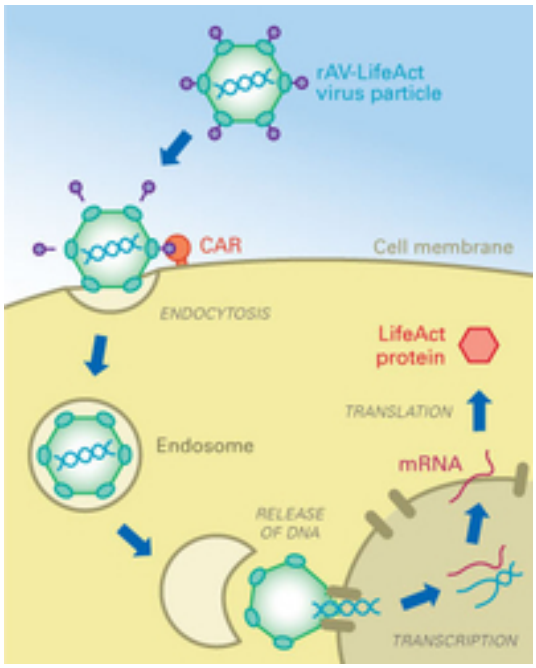

Figure 2: Diagram of transduction of mammalian cells using adenovirus vector from Ibidi cells in focus.

The objective of the current study was to determine whether transduction of bovine granulosa cells via adenovirus with constitutively-active MEK1 augments pERK expression and provides a protective effect against cytokine-induced apoptosis.

2. Materials and Methods

2.1 Cells and culture conditions

Bovine granulosa cells were obtained from slaughterhouse heifer ovaries (Champlain Beef, Inc., Whitehall, NY). Each pair of ovaries collected was treated separately to maintain genotype variation between heifers. Small follicles, defined as three to five millimeters in diameter, were aspirated from the ovaries using a 20-gauge needle and syringe. The granulosa cells were then seeded into T25 flasks, maintained in DMEM/F12 (1:1, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS) and antibiotic/antimycotic (Life Technologies) at 37°C with 5% CO_2 and 95% air with 95% humidity. Once confluent, the cells were seeded into 96-well, 12-well or 6-well plates with media containing 10% FBS. After 24 hours, the media was replaced with serum free media containing ITS (insulin, transferrin, and selenium; 100 ng/mL; Sigma Aldrich) for each experiment.

2.2 Viral transduction of constitutively-active MEK-1 via adenovirus vehicle

Bovine granulosa cells were seeded into 12-well plates at 100,000 or 150,000 cells per well in serum-containing media. Once cells were approximately 70% confluent, media was changed to serum free media supported by ITS. Cells were transduced at the same time with 10, 20, or 50 MOI (multiplicity of infection) of diluted adenovirus containing constitutively active MEK1 (Cell Biolabs, Inc., San Diego, CA) and incubated for 2 hours at 37°C. After that time, serum-containing media (10% FBS) was added and the cells were incubated for an additional 48 hours at 37°C with 5% CO_2 and 95% air with 95% humidity.

2.3 Insulin like growth factor-1 (IGF-1) dose-response

To determine the optimal dose of IGF-1, the cells were seeded into 96-well plates and grown to confluency. Subsequently, they were exposed to 0, 10, 25, 50, or 100 ng/mL of IGF-1 (Cell Signaling, Beverly, MA) for 48 hours. After this incubation period, the cells were exposed to 120 mL of MTS reagent (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega) for one hour at 37°C prior to reading the absorbance at 490 nm on a plate reader (BioTek, model EL808). The MTS assay is a colorimetric method used to measure mitochondrial activity, which is proportional to cell viability in culture.

2.4 Cytokine-induced apoptosis

Bovine granulosa cells were seeded into 12-well plates and transduced with adenovirus as described above. The media was supported with ITS (100 ng/mL) and IGF-1 (100 ng/mL), determined as the optimal concentration in the above experiment. After the 48-hour incubation with the adenovirus, the cells were treated with either Interferon-gamma (IFNγ; Thermo Fisher Scientific, Inc.) at 5 μg/mL or Fas ligand (FasL; EMD Millipore) at 10 μg/mL for 3 hours. Currently, this experiment has been done once.

2.5 Epidermal Growth Factor (EGF) induced upreguation of MEK1 protein

Bovine granulosa cells were seeded into a 6-well plate at 250,000 to 300,000 cells per well. When the cells were confluent, 5 or 10 ng/mL of EGF (Invitrogen) was added to respective wells for 10 minutes at 37°C. This experiment was repeated twice.

2.6 Immunoblot analysis of MEK1 and pERK protein

Confluent cells from above-described experiments were trypsinized and lysed with complete RIPA buffer (Santa Cruz Biotechnology, Dallas, TX) containing protease and phosphatase inhibitor cocktails (Sigma-Alrich, St. Louis, MO). Cells containing RIPA buffer were shaken on a thermomixer for 30 minutes and centrifuged for 15 minutes at 4°C at 13xg. The protein-containing aspirate was collected and quantified via BCA assay. This is colorimetric method used to quantify protein concentration based on a standard curve of bovine serum albumin (BSA) ranging from 25 to 2000 µg/mL. The samples of unknown concentration were incubated in A/B reagent (Life Technologies) for 30 minutes at 37°C and read at 540 nm on a plate reader (BioTek, model EL808).

The quantified protein was resuspended in 5X SDS loading buffer and denatured at 100.5°C for 2 minutes. Total cellular proteins at a concentration of 10 to 30 µg were separated by SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes (EMD Millipore; Darmstadt, Germany). The membranes were blocked in 1% BSA in TBS blocking buffer for 3 hours at room temperature. Immunoblotting was performed using primary antibodies to detect MEK1 (rabbit monoclonal MEK1; Cell Signaling; Beverly, MA) and pERK (rabbit monoclonal phospho-p44/42 MAPK ERK1/2; Cell Signaling). Membranes were stripped with stripping buffer (Thermo Scientific; Newington, NH) and reprobed for cyclophilin B, CyPB, (mouse monoclonal CyPB; Santa Cruz Biotechnology) to ensure equivalent protein loading. A LI-COR Odyssey infra-red imaging system was used to detect secondary antibodies (anti-mouse dylight 800 [1:15,000], Cell Signaling and anti-rabbit dylight 680 [1:15,000], Cell Signaling) at 700 and 800 nm wavelengths.

2.7 Analysis and statistics

Independent experiments were defined by separate ovary pairs. Data were analyzed by one-way or two way analysis of variance (ANOVA), followed by a Tukey's post-test for multiple comparisons. Differences among means at a value of $P<0.05$ were considered statistically significant. Immunoblotts were analyzed using Image Studio Lite software to visualize and quantify band signal intensity. Microsoft Excell was used for generation of graphs.

3. Results

3.1 Transduction with AdMEK1 alters bGC morphology

Morphology of the bGCs showed a dose-response relationship to increasing doses of AdMEK1 virus (MOI). Control cells that received no virus grew to a confluent monolayer in culture. As MOI increased from 10 to 20 to 50 in AdMEK1-transduced cells, conversely, the cells "rounded up" and exhibited increased cytoplasmic granularity. These observations suggest that the cells had indeed been transduced by adenovirus.

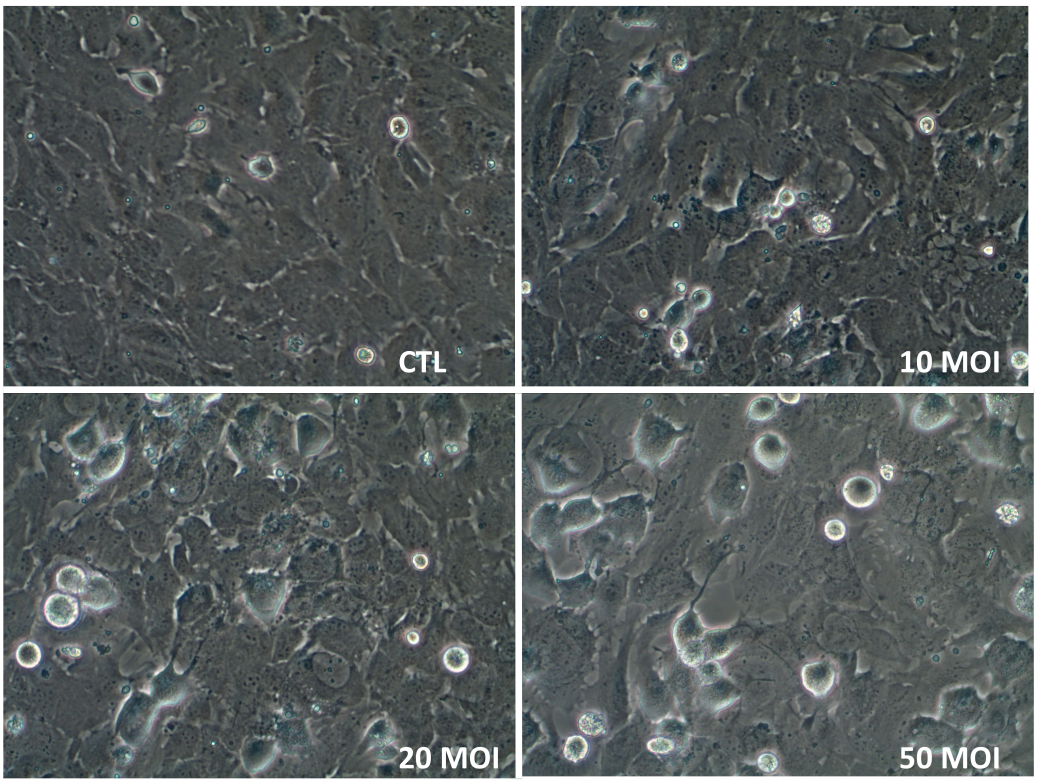

Figure 1. Increasing doses of AdMEK1 (MOI) augmented cytoplasmic granularity and "rounding up" of bGCs 72 hours after viral transduction (20X magnification).

3.2 AdMEK1 transduction fails to enhance MEK1 and pERK expression

There were no overt differences in MEK1 or pERK protein expression in control cultures compared to any AdMEK1-transduced cultures as determined by immunoblotting. Cyclophilin B (CyPB) verified equivalent protein loading across samples (Figure 2). The signal intensity of each band for MEK1 and pERK samples was determined as a ratio to CyPB band intensity, respectively. From this perspective, the relative expression of these proteins did not differ ($P>0.05$) between control and any of AdMEK1-transduced cultures (Figure 3).

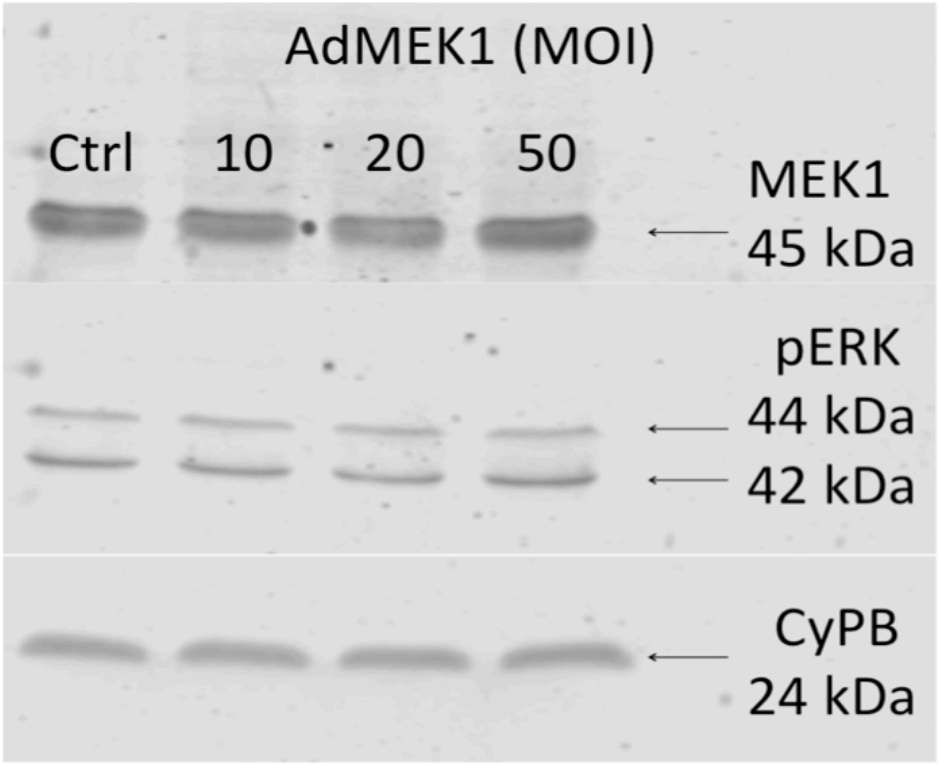

Figure 2. Representative immunoblot of MEK1, pERK, and CyPB expression in control (CTL) and Adenovirus MEK1 (AdMEK1)-transduced bovine granulosa cells. The cells were exposed to doses of 10, 20, or 50 MOI (multiplicity of infection) of AdMEK1 for a period of 48 hours. Immunoblotting revealed no discernable increase in MEK1 or pERK expression at even the highest doses of AdMEK1. CyPB is depicted as the reference protein used to verify equal protein loading among samples.

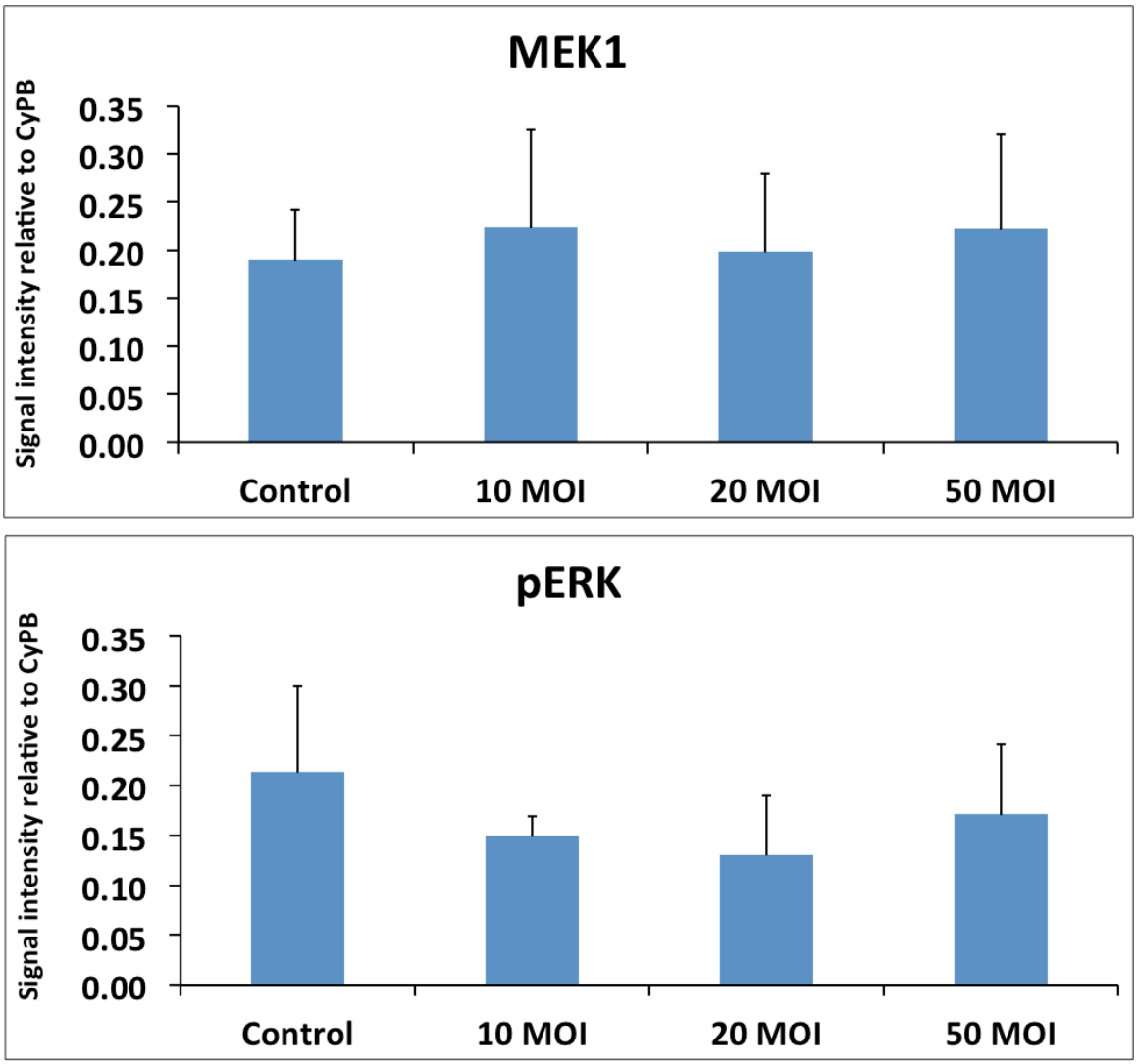

Figure 3: Quantification of signal intensity for MEK1 and pERK protein expression indicated no difference ($P>0.05$) between the control and AdMEK1-transduced bGC cultures (n=4 independent experiments).

3.3 Stimulation of death pathway via cytokines fails to alter MEK1 and pERK expression

Exposure of bGCs to known death-inducing cytokines, Fas ligand (FasL) and interferon gamma (IFNγ), failed to affect MEK1 and pERK expression in AdMEK1-transduced cells. Again, no difference in relative MEK1 or pERK protein expression was observed across treatments. Acknowledging that only one replicate of this experiment was conducted, however, further experimentation will be necessary to confirm these results.

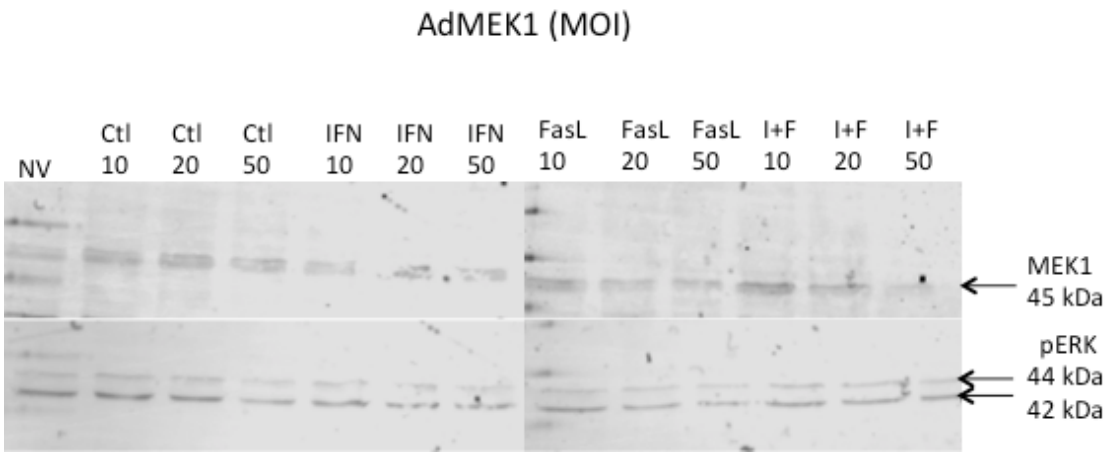

Figure 4. Immunoblot of bGCs exposed to AdMEK1 virus (10, 20, and 50 MOI) in addition to cytokines IFNγ or FasL. Relative MEK1 and pERK protein concentration were not different across treatments (n=1).

3.4 EGF fails to enhance MEK1 and pERK expression

In a separate set of experiments, bGC were exposed to 5 ng/mL or 10 ng/mL EGF for 10 minutes to verify that MEK1 and pERK could be upregulated by a known stimulatory molecule. Surprisingly, there was no readily detectable increase in MEK1 or pERK expression as expected (Figure 5). Image analysis of the blot, however, indicated the 10 ng/ml dose of EGF increased the relative expression of pERK compared to other treatments (Figure 6). Again, additional experiments will be necessary to confirm these findings.

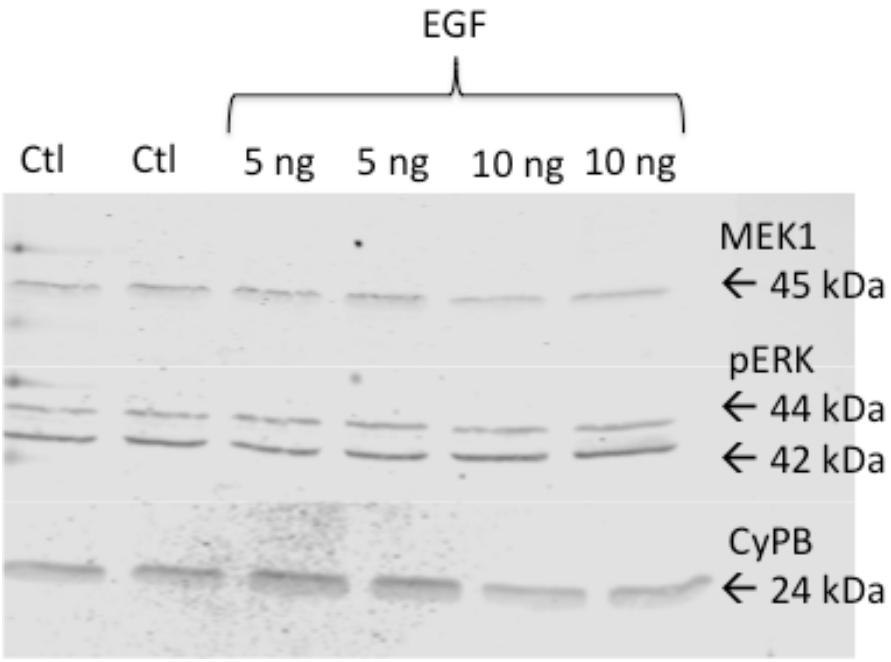

Figure 5. Immunoblotting of bGC proteins revealed no discernable increase in MEK1 or pERK expression following exposure to EGF, as anticipated. CyPB is depicted as the reference protein used to verify equal sample loading among lanes (n=1 experiment).

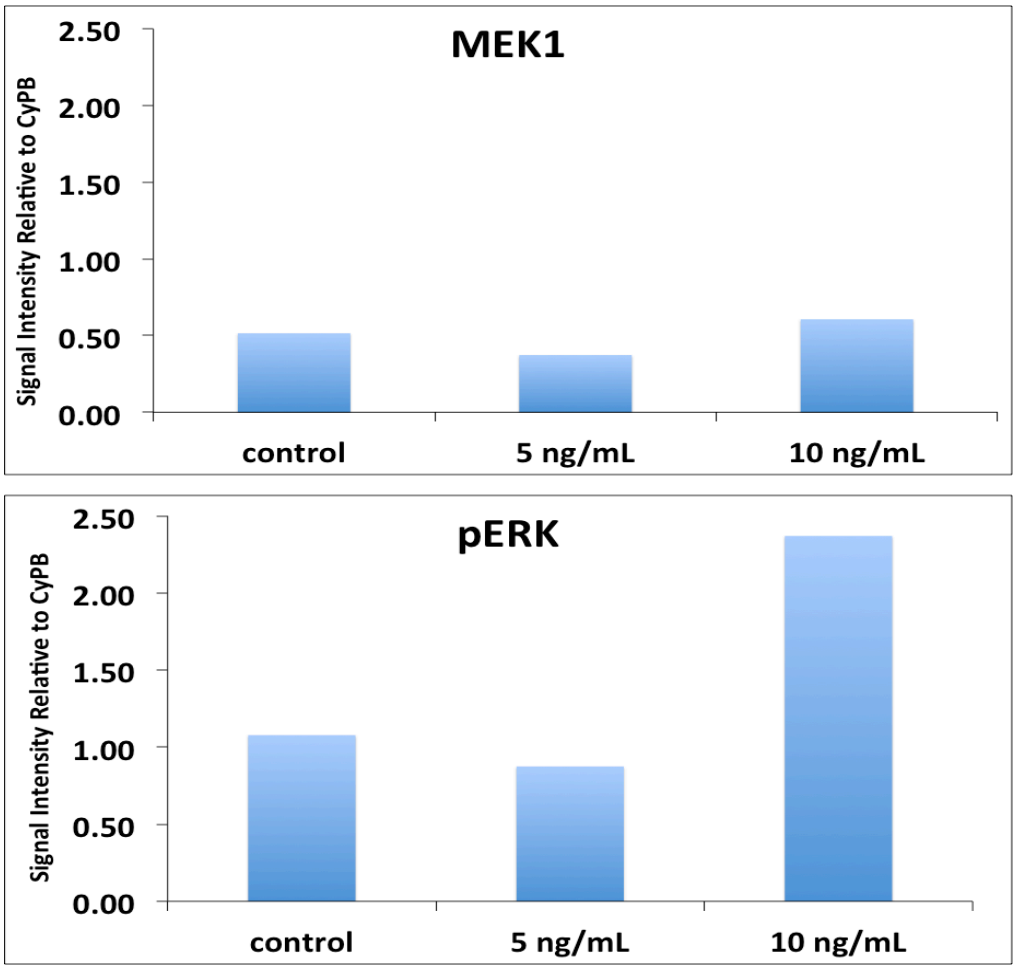

Figure 6. Quantification of signal intensity for MEK1 and pERK protein expression relative to CyPB reference protein (n=1).

4. Discussion

The MAPK pathway is recognized as a growth and pro-survival pathway, and the phosphorylation of ERKs within this pathway is considered a pivotal aspect to the establishment of dominant follicles (Ryan, 2007). However, the mechanisms of these actions remain unclear. The present study was unable to establish consistent upregulation of MEK1 or the phosphorylation of ERKs in bGCs by adenovirus transduction with a

constitutively active MEK1 gene. There are a multitude of explanations to account for the outcomes of the current experiments.

One possibility includes the loss of potency of the virus. The concentration of viral exposure to the cells is measured by multiplicity of infection (MOI), which describes the number of virus particles needed to infect one cell. For example, an MOI of 10 signifies that 10 virus particles are needed to infect one cell efficiently (Ibidi, 2015). However, if the potency of the virus has decreased over time, possibly as a consequence of several freeze-thaw cycles, the calculated MOI and actual MOI may differ. A method we are currently testing to more accurately determine the potency of the AdMEK1 virus is the plaque assay. The principle of this assay takes into account that as adenovirus replication occurs within a specific host cell (i.e., HEK cells), the cells will lyse, forming clear areas or "plaques", which can be visualized following vital dye staining. The size of the plaques is proportional to the relative potency or infectivity of the virus. It is a much more accurate indication of the biological activity of the virus compared to MOI, which is a calculation based upon virus particle concentration (both active and inactive virus particles).

Another explanation for why the virus failed to increase MEK1 or pERK expression could be that the virus was not used at the optimal concentration. If the MOI were too low, then target cells would fail to become infected, and if it were too high, then we would see cytotoxic effects (SignaGen laboratories, 2015). It is clear from the images of the cells that the virus is having an effect on the cells, as they have a change in morphology in a dose-response manner (Figure 1, results). However, as indicated above the plaque assay should provide clearer insight about the correct dose of the virus to be used in future experiments.

Effects of Growth Factors on Cell Proliferation

Growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF), and insulin-like growth factor (IGF) have a role in intraovarian regulation of folliculogensis by enhancing granulosa cell proliferation (Gospodarowicz, 1977; Monniaux, 1997). Signal transduction elicited by EGF in the ovary is associated with rapid phosphorylation of ERKs in a time- and concentration-dependent manner (Keel, 1999). For instance, in freshly isolated porcine granulosa cells (pGCs), EGF induces activation of ERKs within 2.5 to 10 minutes of exposure, with a maximal dose of 10 ng/mL of EGF. The pGCs exhibit a significant upregulation of ERK1/2 and phosphoERK protein (Keel, 1999). In the present study, EGF was used as an experimental positive control to upregulate MEK1 and pERK. Unfortunately, there was little to no evidence that EGF stimulated the expression of these proteins in the current study. This result casts doubt about our ability to detect changes in MEK1 and pERK expression, should they occur.

Nevertheless, there are several possibilities to account for the lack of a EGF-stimulated effect. The bGCs cultured in the current study came from cryopreservation, whereas the pGCs used in the previously-reported study (Keel, 199) were freshly isolated from the ovary. Cryopreservation of cells inevitably leads to post-thaw apoptosis of some cells along with the loss of cellular function in others (Bissoyi, 2014). Thus, cryopreservation of the bGCs might have diminished or impaired their responsiveness to EGF compared to what might be expected of freshly isolated bGCs. This possibility can be explored in subsequent experiments using freshly isolated bGCs.

Overall, the results of the current experiments highlight some of the challenges we encountered when transducing bGCs with AdMEK1. Clear avenues for future exploration

include: using the plaque assay to determine the biological activity/potency of the Adenovirus vector; repeating the EGF experiments to demonstrate physiological upregulation of MEK1 and pERK expression; and eventually testing the hypothesis that this MEK1-pERK signaling pathway provides resistance to cytokine-induced apoptosis in bGCs. Although the evidence of MAPK signaling in ovarian folliculogenesis is clear, its role in bGCs as a mechanism to protect against cytokine-induced apoptosis has not been adequately tested.

5. Acknowledgements

This project was supported by USDA grant 2013-67016-21071 and the UNH Agricultural Experiment Station. Particular appreciation is expressed to Dr. Combelles (Middlebury), Dr. Davis(UNMC), Dr. Tsang, Dr. Walker and their laboratories for providing materials and assistance with the experiments. Appreciation is also expressed to Andraya Ehrlich, Nicole Jaskiewicz, and Sarah Piet for their help with experiments.

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7. Appendix

Appendix I: Adenovirus transduction protocol

Day 1: Seed 12-well plates with 100,000 to 150,000 bGCs per well in
DMEM/F12/10%FBS/Ab

Day 2: Aspirate spent media using vacuum and sterile glass pipette
Change media to serum free: DMEM/F12/ITS/Ab

Day 3: Adenovirus transduction

a. make virus dilutions
   control= DMEM/F12
   50 MOI=.5 µL virus stock + 14.9985 mL DMEM/F12
   20 MOI= 4 mL 50 MOI + 6 mL DMEM/F12
   10 MOI= 2 mL 50 MOI + 8 mL DMEM/F12
b. Add 500 µL of virus dilution to each well
c. Incubate plates for 2 hours (37°C, 5% humidity)
d. Add 500 µL of DMEM/F12/ **20% FBS**/Ab media to each well
   → Final FBS concentration = 10%

Day 5: Collect cell pellets

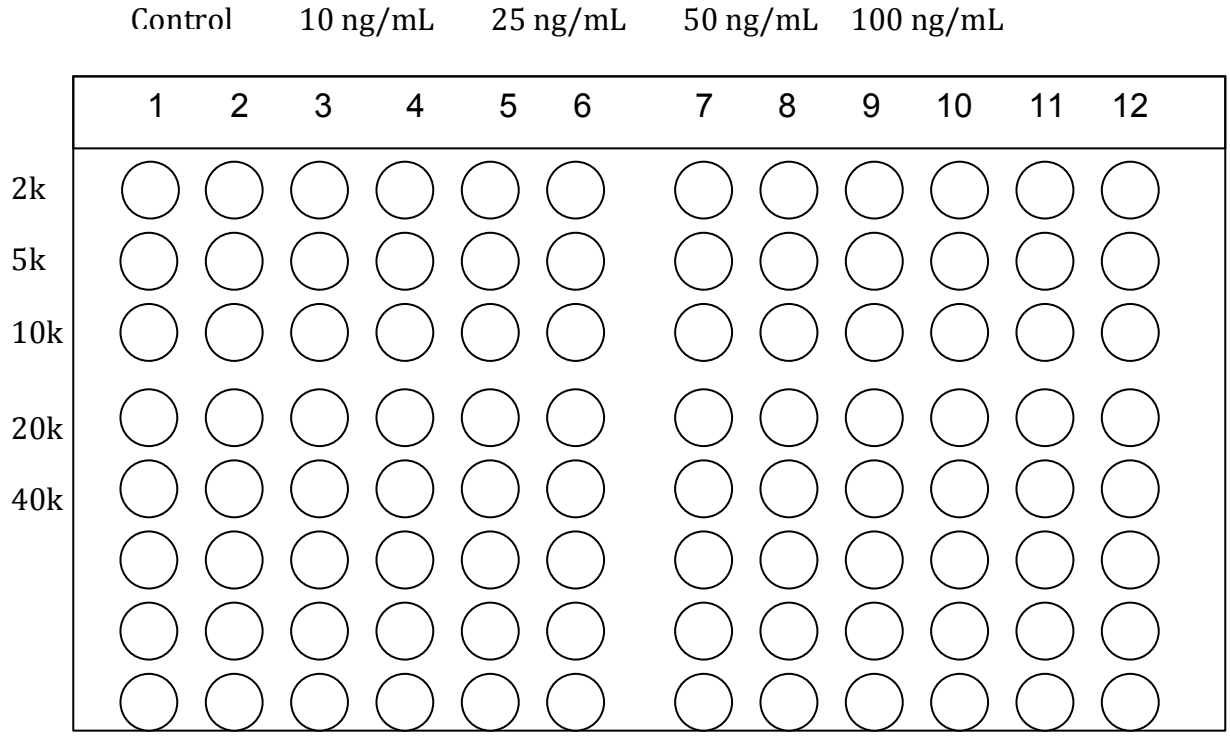
a. Aspirate spent media using pipette and dispensing into bleach
b. Add 500 µL HBSS
c. Remove HBSS
d. Add 500 µL TrypLe
e. Let cells lift (may take up to 20 minutes in incubator)
f. Add 1 mL DMEM/F12
g. Collect cells in 1.5 mL labeled tubes
h. Centrifuge 15 min, 4C, 13xg
i. Remove supernatant and store cell pellets in -80C freezer until protein extraction

Appendix II: Insulin-like growth factor dose-response protocol

Day 1: Seed 96-well plates at 2k, 5k, 10k, 20k, and 40k bGCs per well
DMEM/F12/10%FBS/Ab

Day 2: Aspirate spent media using vacuum and sterile glass pipette
Change media to serum free: DMEM/F12/ITS/Ab

Dose-response to IGF-1
0, 10, 25, 50, or 100 ng/mL
Incubate 48 hours (37°C, 5% humidity)



Day 5: MTS assay

a. Thaw aliquot of liquid reagent to room temperature
b. Add 2 mL reagent to each 10 mL culture medium and mix
c. Place reagent/medium mix into sterile multichannel reservoir
d. Remove conditioned culture medium from 96 well plate by inverting plate on absorbent pad and lightly tapping
e. Add 120 uL of reagent/medium mix to each cell using multichannel pipettor. (Dispense to 1st stop…no air bubbles)
f. Incubate plate (with lid) for 1 hour (37°C, 5% humidity)
g. Read absobance at 490 nm on plate reader (NO lid)
Plate reader should be preceded by 5 sec shaking (medium speed) in plate reader

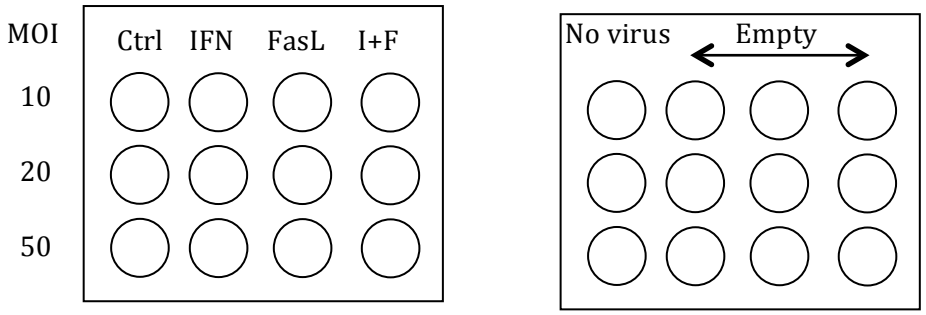
Appendix III: Cytokine-induced apoptosis protocol

Day 1: Seed 12-well plates with 100,000 to bGCs per well in
DMEM/F12/10%FBS/Ab

Day 2: Aspirate spent media using vacuum and sterile glass pipette
Change media to serum free **with IGF-1**: DMEM/F12/ITS/IGF-1/Ab
ITS and IGF-1 at 100 ng/mL
1 mL media per well

Day 3: Adenovirus transduction

e. make virus dilutions
control= DMEM/F12
50 MOI=.5 µL virus stock + 14.9985 mL DMEM/F12
20 MOI= 4 mL 50 MOI + 6 mL DMEM/F12
10 MOI= 2 mL 50 MOI + 8 mL DMEM/F12
f. Add 500 µL of virus dilution to each well
g. Incubate plates for 2 hours (37°C, 5% humidity)
h. Add 500 µL of DMEM/F12/ **20% FBS**/Ab media to each well
→ Final FBS concentration = 10%



Day 5:

a. Media change to DMEM/F12/ITS/IGF-1/Ab (1 mL per well)
b. Add 10 µL spikes of diluted IFN (5µg/mL) to appropriate wells (6 total)
c. Incubate 24 hours at 37°C, 5% CO_2

Day 6:

a. Media change to DMEM/F12/ITS/IGF-1/Ab (1 mL per well)
b. Add 10 µL spikes of FasL (10µg/mL) & diluted IFN (5µg/mL) to appropriate wells
c. Incubate 3 hours at 37°C, 5% CO_2.
d. Collect cell pellets (see appendix I, Day 5)

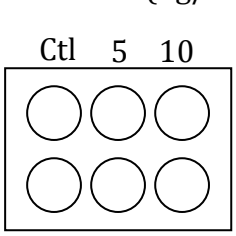
Appendix IV: Epidermal growth factor induced upregulation protocol

Day 1: Seed bGCs into 6-well plate at 300,000 cells/well

Day 3: Aspirate spent media
Change to serum-free= DMEM/F12/ITS/Ab

Day 4: EGF treatment and collect cell pellet

a. Change media= DMEM/F12/ITS/Ab (1mL per well)
b. Add 10uL spike of EGF (5 ng/mL or 10 ng/mL)
c. Incubate 10 min (37°C, 5% humidity)
d. Collect cell pellets (see appendix I, Day 5)



Appendix V: Protein extraction and quantification protocol

Protein Extraction Protocol

Materials:
RIPA Lysis Buffer Kit (Santa Cruz Biotech. # sc-24948)
Pre-chilled thermomixer
Pre-chilled centrifuge
Sigma P8340 Protease Inhibitor Cocktail 1:100
Sigma P5726 Phosphatase Inhibitor Cocktail 1:100

3 ml complete RIPA per gram of tissue OR 1 ml complete RIPA per $2.0x10^7$ cells in suspension OR 0.6 ml complete RIPA per subconfluent monolayer on a 100 mm plate.

Protocol:
On ice prepare the RIPA Lysis Buffer:

1X RIPA	1 ml
Protease Inhibitor Cocktail	10 µl
Phosphatase Inhibitor Cocktail	10 µl
Phosphatase Inhibitor Cocktail	10 µl

Complete the following steps on ice or using pre-chilled instruments:

1. Add 75-100 µl compete RIPA to each tube.
2. Transfer to a 1.5 ml tube
3. Pass through 20-gauge needle 10-20X; remove clumps as needed.
4. Shake in thermomixer for 30 min at 1000 rpm at 4°C (cold room).
5. Pass through 20-gauge needle 10-20X; remove clumps as needed.
6. Spin samples at 14000 x g for 15 min at 4°C.
7. Transfer supernatant into fresh 1.5 ml tube..
8. Quantitate protein concentration and aliquot, places tubes at -80°C.

Protein Quantification Protocol: BCA Protein Assay

Smith et al. Anal. Biochem. 150, 76-85 (1985).
Adapted from Pierce Chemical Co. "BCA Protein Assay Reagent Kit".

Materials:

1. BCA reagents A and B. (Pierce Chemical Co., Kit # 23225).
2. Microtiter plates.
3. Glass 12x75mm test tubes.
4. Microtiter plate reader: 540-590nm.
5. 2000 µg/mL solution of bovine albumin in water or buffer identical to samples.

Working Reagent:

Mix 50 parts reagent A to 1 part reagent B. When reagent B is initially added to reagent A, the solution is turbid and yields a clear green color upon mixing. Prepare sufficient amount of working reagent: each microtiter well requires 200µL.

Procedure:

1. Prepare standards in glass test tubes, according to the following table:

Tubes	BSA Standard	Water/Buffer	µg Protein
Stock	300 µL stock	0 µL	2000 µg/mL
A	375 µL stock	125 µL	1500 µg/mL
B	325 µL stock	325 µL	1000 µg/mL
C	175 µL (A)	175 µL	750 µg/mL
D	325 µL (B)	325 µL	500 µg/mL
E	325 µL (D)	325 µL	250 µg/mL
F	325 µL (E)	325 µL	125 µg/mL
G	100 µL (F)	400 µL	25 µg/mL

2. Place 10µL of each standard or unknown sample into the appropriate microtiter plate wells. Use 10µL of the diluent for the blank wells.
    a. Pipet 10µL of standards in duplicate in columns 1 and 2.
    b. Pipet 10µL of samples into pairs of wells across the plate. For example, row C: columns 3 and 4, 5 and 6, etc.
3. Mix 50 parts of Reagent "A" (e.g. 3.5mL) with 1 part Reagent B (e.g. 70µL).
4. Add 200µL of the working reagent to each well, mix the plate well on a plate shaker for 30 seconds.
5. Cover the plate and incubate the plate at 37ºC for 30 minutes.
6. After incubation, cool the plate to room temperature.
7. Measure the absorbance at or near 562nm on a plate reader.
8. Subtract the average A(562) reading for the blanks from the A(562) reading for each standard or unknown sample.
9. Prepare a standard curve plotting the average blank corrected A(562) reading for each BSA standard versus its protein concentration in µg/mL. Using the standard curve, determine the protein concentration for each unknown sample

Appendix VI: Immunoblotting and immunodetection protocols

Adapted from Mini-PROTEAN Tetra Cell Gel Apparatus

ELECTROPHORESIS

1. Prepare acrylamide gel according to Mini-PROTEAN Tetra Cell Gel Apparatus directions
2. Remove polymerized gel from castor and secure into clamping frame
3. Sample loading:
    a. fill upper chamber with tank buffer to just under the edge of the outer gel plate
    b. put lane outlets on glass to see where lanes are
    c. dilute protein with 5X running buffer
    d. put lysates on heating block for 2 min at 100.5C, vortex, spin down, and keep on ice until loading
    e. slowly load samples into each of the assemblies with gel loading tips
4. Electrode assembly in tank and gel removal:
    required total buffer volume= 700 mL for 2 gels, 1000 mL for 4 gels
    a. place tank on flat surface, with the front facing you
    b. fill lower chamber with buffer to the indicated level (550 mL for 2 gels, 680 mL for 4 gels)
    c. place the lid on the tank, being sure to align the color-coded plugs and jacks
    d. Insert the electrical leads into power pack and turn on the switch in the back
    e. Turn the timer to 1 hour and set the current to 20 for 1 gel and 40 for 2 gels
    f. When samples have run off the gel, turn off the power pack and disconnect the electrical leads
    g. Remove tank lid and pour off running buffer
    h. Remove the gels from the gel cassette by gently separating the two plates

TRANSER OF PROTEINS TO A MEMBRANE

a. Cut off the stacking gel and upper left corner of the gel to know orientation
b. Rinse gel with ddH2O and place it in a pyrex dish with transfer buffer for 15 min
c. Measure the gel and cut 6 pieces of blotting paper and a piece of cellophane that size, per gel
d. For PVDF membrane, place it in 100% methanol for 15 sec, rinse with ddH2O, and place it in transfer buffer
e. Tape down a slot mylar mask in transfer box
f. Place the following down in the proper order:
    3 pieces of blot paper dampened with transfer buffer
    membrane
    gel *only get once chance, cannot move it once its down
    3 pieces of damp blot paper
g. Place cover on transfer box and plug the electrode into itself
h. Plug black and red electrodes into power pack, one on top of the other
i. With voltage all the way clockwise and current all the way counterclockwise, turn it on for 45 min, set current to 36

IMMUNODETECTION

a. remove membranes and gels from transfer box
b. put gel into container with staining solution on a rocker overnight
c. put PVDF membrane into 100% methanol for 15 sec, rinse with ddH2O, and put into blocking buffer for 3 hours on a rocker at room temperature
   1% BSA in TBS, filtered
d. after 3 hours, pour blocking buffer back into its container and store in freezer
e. pour the primary antibody on the membrane, float a piece of parafilm over the antibody/membrane, cover
f. incubate overnight at 4C on rocker in 4th floor walk-in refrigerator
g. pour primary Ab back into its container and store in freezer
h. wash membrane with TBST 2x rapidly, 1x for 15 min, and 3x for 5 min on rocker
i. incubate membrane in secondary antibody for 1 hour at room temp on rocker
   *keep DARK, use aluminum foil and overlay with parafilm
j. pour secondary antibody back into its container and store in freezer
k. wash again, same as above
l. put membrane in "membrane drying box" and bring it to the Odyseey LiCor

Appendix VII: Undergraduate Research Conference Poster, April 24th 2015
Parents Association Symposium Award of Excellence

Influence of Extracellular-Regulated Kinases (ERKs) on Granulosa Cell Fate in the Bovine Follicle

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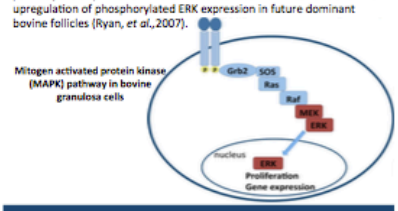
Abstract

Each year, infertility in cows costs the dairy industry over $400 million. This project is focused on the onset of follicular atresia in cows; the process by which the egg-bearing follicles die prematurely and can lead to infertility. The cellular mechanisms that influence programmed cell death, or apoptosis, of bovine granulosa cells (bGCs) within follicles are being investigated. In general, the extracellular regulated kinases (ERKs) are intracellular signaling proteins believed to influence the proliferation, survival, and differentiation of cells. In the context of follicular atresia, however, the relationship between the onset of apoptosis of bGCs and the effects of ERKs is relatively unknown. In the present study we are utilizing a constitutively active mitogen activated protein kinase (MEK1) to upregulate ERKs in bGCs, and then determine the effect on bGC sensitivity to apoptosis. Cultured bGCs were genetically modified via transduction using a constitutively active MEK1 adenovirus. Currently we are verifying the efficiency of viral transduction, overexpression of MEK1, and downstream activation of ERK by flow cytometry and immunoblotting. Preliminary results indicate a lack of MEK1 up-regulation in transduced bGCs. Subsequent experiments will focus on optimizing adenovirus transfection in order to test the hypothesis that upregulated ERKs prevent cytokine-induced apoptosis of bGC.

Introduction

Apoptosis of granulosa cells is a characteristic of the onset of ovarian follicular atresia. It is proposed that vulnerable cells lack intrinsic pro-survival signaling mechanisms. The mitogen activated protein kinase pathway (MAPK; see below), the pathway investigated in this study, is understood to be a pro-survival pathway. The downstream ERKs of this pathway are of particular interest because recent studies have shown an upregulation of phosphorylated ERK expression in future dominant bovine follicles (Ryan, *et al.*, 2007).



Objective

Investigate the effects of MEK1 upregulation in bovine granulosa cells

Materials and Methods

1. Established cultures of bovine granulosa cells (bGCs)
   - Collected bovine ovaries from slaughter house (Champlain Beef, Inc., Whitehall, NY)
   - Aspirated small follicles (3-5 mm)
   - Cultures maintained in DMEM/F12/10% FBS/Antibiotic at 37°C, 5% CO_2 in air
2. Transduced bGCs with constitutively-active AdMEK1 virus (Cell Biolabs, Inc.) at 10, 20, or 50 MOI (multiplicity of infection) for 48 hours
3. Lysed bGCs with RIPA buffer and quantified protein concentration via BCA assay
4. Verification of expression of MEK1 or pERK via immunoblotting

Results

Transduction with AdMEK1 alters granulosa cell morphology

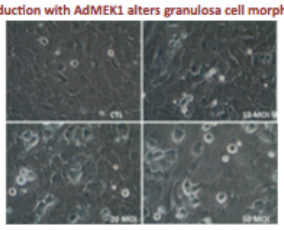


Figure 1. Increasing doses of AdMEK1(MOI) augmented cytoplasmic granularity and "rounding-up" of bGCs 72 hrs after viral transduction (20X magnification).

AdMEK1 transduction fails to enhance MEK1 and pERK expression

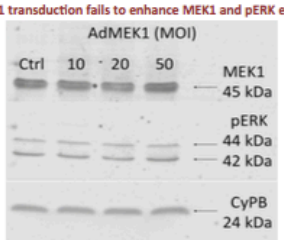
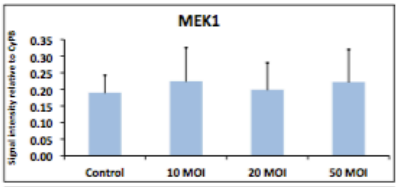


Figure 2. Despite morphological changes observed in culture, immunoblotting of bGC proteins revealed no discernable increase in MEK1 or pERK expression at even the highest doses of AdMEK1. Cyclophilin B (CyPB) is depicted as the reference protein used to verify equal sample loading among lanes.

Results (continued)

AdMEK1 transduction fails to enhance MEK1 and pERK expression



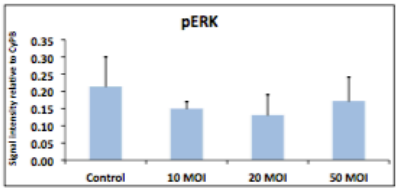


Figure 3: Quantification of signal intensity for MEK1 and pERK protein expression indicated no difference (P>0.05) between the control and virally-transduced bGC cultures (n=3 independent experiments).

Summary

- AdMEK1 transduction affected bGC morphology *in vitro* in a dose-dependent manner that resulted in increased cellular granularity and rounding.
- However, no upregulation of MEK1 or pERK expression was evident in virally-transduced cells.
- Future work: determine if virally-transduced cells are resistant to death inducing/apoptotic mechanisms.

Acknowledgements:
This project was supported by USDA grant 2013-67016-21071 and the UNH Agricultural Experiment Station. Particular appreciation is expressed to Dr. Combelles (Middlebury), Dr. Davis (UNMC), Dr. Tsang, Dr. Walker and their laboratories for providing materials and assistance with the experiments.

Reference:
Ryan KE, Casey SM, Canty MJ, Crowe MA, Martin F, Evans AC. Akt and Erk signal transduction pathways are early markers of differentiation in dominant and subordinate ovarian follicles in cattle. Reproduction 2007; 133:617-626.

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