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Regulation of Angiogenesis by Insulin-Like Growth Factor in the Bovine Corpus Luteum: A Preliminary Study

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Keywords
angiogenesis, IGF-1, CCN1, corpus luteum, MMP, bovine

Subject Categories
Biochemistry | Cellular and Molecular Physiology | Endocrinology | Molecular Biology

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Abstract

In the ovary, growth of a new vascular network, or angiogenesis, is a hallmark of corpus luteum (CL) development. This process requires several angiogenic factors and matrix metalloproteinases (MMPs). Recently, we reported the expression of an angiogenic inducer, **C**ysteine rich 61-**C**onnective tissue growth factor-**N**ephroblastoma overexpressed (CCN1), in the bovine CL. However, it is not known how CCN1 is regulated in the CL. Therefore, the goals of the present study were 1) to determine the regulation of CCN1 by insulin-like growth factor (IGF-1) during the early (Day 4), mid (Day 8), and late (Day 16) stages of CL development, and 2) to determine the effect of IGF-1 on MMP 2 and 9, both of which are involved in angiogenesis. Preliminary results showed that CCN1 was rapidly induced within 2 to 4 hours. As such, luteal cells were treated for 2 hours with IGF-1 (50 ng/mL and 100 ng/mL). Analysis via quantitative PCR (qPCR) suggests that treatment with IGF-1 may increase CCN1 expression in the mid and late stage CLs. Zymography results suggest that IGF-1 may increase MMP 2 expression in mid and late stage CL and MMP 9 expression in the early and mid-stage CL. These experiments will be repeated to confirm observations.

Keywords: angiogenesis; IGF-1; CCN1; corpus luteum; bovine; qPCR; zymography; MMP

Literature Review

The Bovine Ovarian Cycle

In the Unites States, it is estimated that the dairy industry loses $400 million annually due to embryonic and fetal loss (1). There are many factors that contribute to this loss, including sub-optimal growth of oocytes within follicles, a decrease function of the corpus luteum, and a hostile uterine environment that does not promote implantation of the embryo. Due to its role in maintaining pregnancy, the ovarian gland called the corpus luteum (CL) was the main focus of this study.

The CL develops after ovulation of the dominant follicle, however, before ovulation can occur, the ovary must first prepare the follicle to be released. This process is known as folliculogenesis, in which an immature ovarian follicle, containing a primary oocyte and surrounding granulosa cells, develops into a mature, dominant follicle that is a destined for ovulation (2).

Folliculogenesis begins with production of follicle stimulating hormone (FSH) from the anterior pituitary. FSH stimulates the proliferation of the granulosa cells that surround the primary oocyte within the follicle, which aids in the transformation of a primordial follicle into a primary, secondary, and finally a tertiary, or antral follicle (2). The antral follicle is characterized by a fluid filled antrum within the basement membrane and an outer vascularized layer of theca cells surrounding the follicle.

As FSH stimulates proliferation of the granulosa cells, luteinizing hormone (LH) induces the production of androgens (e.g. androstenedione and testosterone) in the ovary. LH binds to LH receptors on the theca cells stimulating production of testosterone, which is then converted to estradiol, by the FSH-induced aromatase in granulosa cells (2). Then, estradiol triggers the

hypothalamus to secrete gonadotropin-releasing hormone (GnRH), which triggers a rise in the secretion of LH.

As the follicles develop, the granulosa cells begin to secrete inhibin, which inhibits FSH production in the anterior pituitary (3). With a decline in FSH levels, the developing follicles must compete for FSH binding. The follicles that receive more FSH will proliferate, and begin to secrete more estradiol as they become more dominant (2). Eventually one follicle will dominate, and it is this dominant or Graafian follicle, with its elevated estradiol production, that will trigger a LH surge through a positive feedback loop via the hypothalamo-hypophyseal-ovarian axis.

The surge of LH signals the ovary to prepare for ovulation by 1) increasing the follicular pressure in the antral follicle and 2) weakening the outer connective tissue, the tunica albuginea. LH increases the follicular pressure by upregulating the production of prostaglandin E_2 (PGE_2) (4) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (5). PGE_2 increases the blood flow to the ovary and the dominant follicle, which results in edema inside the ovary, while $PGF_{2\alpha}$ increases the contraction of the smooth muscle in the ovary. The Graafian follicle is filled with fluid that when under increased pressure, from contractions and edema, ruptures and releases the oocyte from within.

However, in order for the oocyte to leave the follicle, the tunica albuginea must be degraded. LH aids in the degradation of the follicle wall by upregulating $PGF_{2\alpha}$ and proteolytic enzymes. $PGF_{2\alpha}$ stimulates the release of lysosomal enzymes that degrade the follicular wall and proteolytic enzymes, like matrix metalloproteinase (MMP)-1 (collagenase) and MMP-2 (gelatinase), which degrade the tunica albuginea (6). The combined degradation results in a weakening of the ovarian surface that will rupture and allow for the release of the oocyte during ovulation (2).

Luteal Phase, Corpus Luteum Formation, and Progesterone Production

The LH surge that resulted in ovulation also plays a role in transitioning the follicle to become the corpus luteum. LH receptors are present on theca cells and they are upregulated on granulosa cells by estradiol during folliculogenesis (7). When LH binds to their receptors, a variety of physiologic changes within the granulosa and theca cells follow (7). First, LH decreases the production of cyclin dependent kinase (cdk)-2, a positive cell cycle regulator, and increases the production of p27 and p21, two cdk inhibitors, which result in the granulosa and theca cells being arrested at the G_0/G_1 phase of the cell cycle (7). Second, LH upregulates the expression of a multitude of steroidogenic related proteins within granulosa and theca cells, including steroidogenic acute regulatory protein (StAR), cholesterol side-chain cleavage enzyme (CYP11A1; P_{450}scc), and 3β-hydroxysteroid dehydrogenase (3βHSD) (7). Lastly, LH down-regulates FSH receptors on the granulosa cells, which initiates the switch in hormone production from estradiol to progesterone (2). Overall, LH transforms the granulosa and theca cells of the follicle to steroidogenic large and small cells of the corpus luteum, respectively.

After ovulation, the Graafian follicle collapses and the basement membrane that once divided the avascular granulosa compartment from the vascular theca compartment degrades (1). The granulosa and theca cells have already begun LH-induced transformation into luteal cells; these steroidogenic cells along with fibroblasts, smooth muscle cell, and immune cells form the CL (8).

The formation of the CL, and more specifically the degradation of the basement membrane, also allows new blood vessels to sprout from parent vessels in the theca compartment (a process known as angiogenesis) and to infiltrate the granulosa cell compartment (1). This newly formed blood vessel network allows the CL to receive one of the greatest rates of blood flow in the body,

which is essential for transport of oxygen and nutrients, but most importantly cholesterol. The major function of the CL is progesterone production, which is derived from cholesterol. Cholesterol is transformed to pregnenolone by CYP11A1, which causes a hydroxylation at the C-20 and C-22 carbons that results in the cleavage of the side chain (9). Pregnenolone is then converted into progesterone by the oxidation of a C-3 to a keto group by 3βHSD. This causes a double bond to shift into the conjugated position and results in the formation of C_{21} progesterone (9).

Progesterone production is essential because it is required for the establishment and maintenance of pregnancy (1). Inadequate amounts of progesterone compromise uterine endometrial growth and embryo survival; therefore, pregnancy cannot be maintained. The process of angiogenesis is crucial in the production of progesterone and also in the distribution of progesterone to other parts of the body (10). Thus, without angiogenesis and a rich blood vessel network in the CL, progesterone production and maintenance of pregnancy would be compromised.

Angiogenesis in the Bovine Corpus Luteum

In the early stage CL, the rate of angiogenesis is at its highest, and the proliferation of capillaries results in a dense network of vasculature that infiltrates the CL (11). In the mid stage CL, angiogenesis is still ongoing (angiomaintenance) in order to maintain the vasculature. At this point in the luteal phase, nearly every luteal cell is in contact with a capillary, thus insuring that the produced progesterone is distributed to the body (12). In the late stage CL, it is beginning to undergo luteolysis, as does the vasculature via angioregression. The development and regression of the corpus luteum is closely tied to the development and regression of its vasculature.

In the bovine CL, there are several known angiogenic inducers and factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF2), IGF-1, and CCN1. VEGF, a common and potent angiogenic factor, is produced by luteal cells. It is a mitogen for endothelial cells, and it stimulates vascular permeability. Likewise, FGF2 is also produced by luteal cells and it signals endothelial cell proliferation, migration, and differentiation (12). The present study focused on the regulation of the angiogenic inducer, CNN1, and two members of the matrix metalloproteinase (MMP) family, MMP 2 and MMP 9, by IGF-1.They are described in more detail below.

Insulin-Like Growth Factor 1

Insulin-like growth factor (IGF)-1 is a member of a family of insulin-related peptides. It is composed of 70 amino acids and has a molecular weight of 7649 Da (13). Like insulin, IGF-1 is composed of an A and B chain connected by disulfide bridges; however, unlike insulin, IGF-1 also has a 12 amino acid long C chain that it retains. Due to its similarity with insulin, IGF-1 is able to bind to insulin receptors with low affinity (13), along with its own receptor. The IGF-1 receptor is a heterotetramer composed of two extracellular α subunits linked by disulfide bonds and two transmembrane β subunits that are linked to the α subunits by disulfide bonds as well (13). IGF-1 binds to α subunits and causes a conformational change that results in autophosphorylation of tyrosine kinases that make up part of the intracellular domain of the β subunits (13). IGF-1 bound to its receptor can initiate many signaling cascades that involve insulin receptor substrate 1 (IRS-1), growth factor receptor bound protein 2 (Grb2), src homology domain protein (Shc), and phosphatidylinositol 3 kinase (PI3 kinase) (13).

Insulin-like growth factor has an important role in the ovary because it has been implicated to stimulate progesterone production, induce proliferation of granulosa cells, and prevent apoptosis. In a study by Ptak et al. (2003), they showed that administration of IGF-1 to porcine luteal cell upregulated the synthesis of steroidogenic enzymes, like 3βHSD, which increased progesterone production in early stage CLs (14). In another study by Hu et al. (2004), they showed that IGF-1 upregulates the phosphorylated protein kinase B (pAkt) and mitogen-activated protein kinase (MAPK) pathways in the granulosa cells, which results in cell proliferation and protection against Fas-induced apoptosis (15).

Along with progesterone production and granulosa cell survival, IGF-1 has also been implicated in processes associated with new blood vessel formation in the bovine CL (16). In a study by Sarkissyan et al. (2014), they found that IGF-1 increases CCN1 levels in breast cancer cells, which resulted in increased proliferation and migration (16). This study identified pAkt and MAPK as being potential candidates for IGF-1 signaling. The binding of IGF-1 to its receptor can signal either Grb2 or IRS-1 to bind to the phosphorylated tyrosine kinases on the β subunits of the IGF-1 receptor. Grb2 initiates the MAPK signaling cascade, whereas IRS-1 initiates the PI3 kinase cascade that ultimately results in the activation of pAkt (13).

Mechanistically, Sarkissyan et al. (2014) determined that constitutively expressed pAkt upregulated CCN1 expression, but when pAkt was blocked, IGF-1-induced CCN1 expression was not abolished. Interestingly, inhibition of MAPK decreased CCN1 expression, while addition of IGF-1 did not restore its expression. Overall, in breast cancer cells, IGF-1 induction of CCN1 expression is dependent on pAkt and MAPK signaling. Whether or not IGF-1 and CCN1 engage similar signaling pathways in other reproductive tissues, e.g. the ovary, is currently not well understood.

CCN1

The CCN1 protein family members have four structural domains; 1) a domain highly homologous to the insulin-like growth factor binding protein (IGFBP); 2) a domain homologous to the von Willebrand factor type C (vWC); 3) a thrombospondin type-1 repeat (TSP) domain; and 4) a cysteine knot-containing (CT) domain, plus an N-terminal secretory signal for

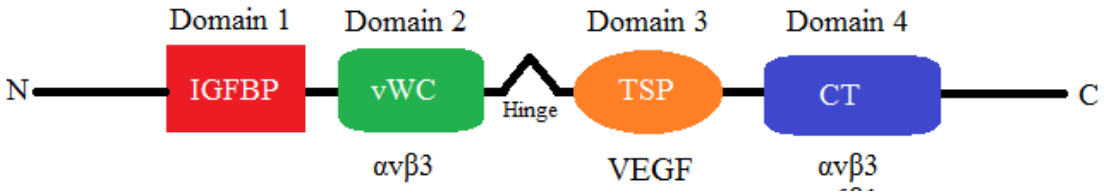

***Figure 1**:* The CCN1 binding domains and the corresponding integrins associated with angiogenesis.

extracellular release (Figure 1) (17). Each CCN1 molecule modulates the extracellular matrix (ECM) and influence cell signaling by binding to heterodimeric integrins (α and β subunits). The multitude of α and β subunit combinations allows for CCN1 to have many physiological effects, including cell adhesion, migration, and proliferation (17). For angiogenesis, the vWC and CT domains of CCN1 bind to the integrins, αvβ3 and α6β1, to promote endothelial cell migration and proliferation (17), resulting in new blood vessel formation in the CL.

The TSP and CT domains of CCN1 are pivotal in the process of angiogenesis; both domains interact with VEGF, an endothelial cell mitogen that stimulates vascular permeability (18). A previous study by Zhang et al. (2001) showed that CCN1 is expressed in the bovine CL, and its upregulation in the young CL is correlated with the switch to angiogenesis following ovulation (11). This study also proposed that there is an interaction between CCN1, VEGF, and FGF2 that promotes the high rate of angiogenesis associated with the early formation of the CL (11). Being a secreted extracellular matrix-associated protein, CCN1 promotes angiogenesis

through endothelial cell adhesion and migration, and through growth factor induced DNA synthesis (19).

Matrix Metalloproteinases

For angiogenesis to occur, one of its earliest stages is to clear away the ECM surrounding the luteal cells with proteolytic enzymes, such as the MMPs. The MMP family consists of 25 different zinc- and calcium-dependent endopeptidases that degrade all known components of the ECM (20). In a previous study by Towle et al. (2002), they showed that two members of the MMP family, MMP-2 and MMP-9, are produced by luteal cells (21). Due to these results, this study chose to focus on MMP-2 and MMP-9 and their relationship to IGF-1.

MMP-2 (72-kDa type IV collagenase or gelatinase A) and MMP-9 (92-kDa type IV collagenase or gelatinase B) consist of two zinc ions, 2-3 calcium ions, and a fibronectin-like domain (16-17). The catalytic zinc ion is necessary for the proteolytic activity of MMP-2 and MMP-9, the calcium ion is required to stabilize the enzyme, and the fibronectin-like domain is essential for binding of MMP-2 and MMP-9 to the basement membrane collagen (22). All these domains work synchronously to allow the MMPs to degrade the ECM for tissue remodeling. In the bovine CL, the degradation of the ECM is necessary in angiogenesis for two reasons. First, the MMPs clear away the ECM surrounding the luteal cells to allow for neovascularization of the CL (7). Second, the degradation of the ECM by MMP-2 and MMP-9, is believed to liberate matrix-bound proteins, like VEGF (23) and CCN1, making them available to interact (24) with endothelial cells and promote migration, which ultimately results in angiogenesis.

Overall, there are many regulators of angiogenesis in the bovine CL: VEGF, FGF2, CCN1, IGF-1, and MMPs. In previous study by Zhang et al. (2001), an interaction between

CCN1, VEGF, and FGF was proposed to promote the high rate of angiogenesis associated with early formation of the CL (11). However, IGF-1 was not mentioned. We know IGF-1 plays an important role during the luteal phase, as it is responsible for upregulating progesterone production, and it is believed to have a role in angiogenesis as well (14) (15) (16). Although IGF-1 has been implicated in the upregulation of CCN1 in breast cancer cells, the role of IGF-1 in regulating CCN1 expression in the bovine CL is currently not well understood. Therefore, the aim of the present study was to answer the following questions: Does insulin-like growth factor 1 (IGF-1) regulate angiogenesis in the bovine corpus luteum? Specifically, does insulin-like growth factor 1 (IGF-1) regulate CCN1, MMP-2 and MMP-9 expression in three developmental stages of the bovine CL?

Experimental Procedures

Corpus Luteum Tissue Collection

All animal procedures were performed according to Institutional Animal Care and Use Committee (IACUC) approved protocols. Corpora lutea were collected from regularly cycling dairy cows at the University of New Hampshire's Fairchild Dairy Teaching and Research Center. The mid and late stage corpora lutea were enucleated from the ovary, whereas the early stage corpus luteum was removed by colpotomy from the dairy cow under an epidural anesthesia (2% mepivacaine hydrochloride).

Luteal Cell Culture

Corpora lutea were obtained from regularly cycling dairy cows at the early (day 4), mid (day 8), and late (day 16) luteal stages of development. The luteal tissue was minced, and placed in a spinner flask containing crude collagenase type 1 in Ham's F12 medium containing 0.05% bovine serum albumin (BSA) for two 1-hour dissociations. During each dissociation, the tissues were triturated every 10 minutes. After completing each dissociation, the supernatant was sequentially centrifuged at 190 G forces, 110 G forces, and 80 G forces to obtain steroidogenic luteal cells. Cell viability was assessed by trypan blue exclusion and cell number was determine with a hemocytometer and verified using a cell counter.

The dissociated luteal cells were seeded ($1x10^6$ cells/well or $0.5x10^6$ cells/well) in 6-well plates containing Ham's F12 medium supplemented with sodium bicarbonate (7.5%), gentamicin (30ug/mL: Gibco-BRL), and insulin/selenium/transferrin (100X) (ITS; Sigma). Cells were incubated in an atmosphere of 95% air/5% CO_2 at 37°C. After 24 hours, unattached cells and red blood cells were removed by rinsing with fresh medium. After an additional 24 hours, the pre-treatment medium was collected and the cells were treated for 2 hours with medium containing 10% fetal bovine serum (FBS; positive control) or IGF-1 (50ng/mL or 100ng/mL). After 2 hours, the post-treatment medium was collected and saved for later analysis.

Quantitative PCR (qPCR)

Ribonucleic acid (RNA) was isolated from the treated luteal cells using RNeasy kits (Qiagen) according to the manufacturer's instructions. A SuperScript VILO (Invitrogen) kit was used to generate cDNA. Using qPCR, expression of mRNA was determined with SYBR Green Master Mix (Applied Biosystems) and assessed using the QuantStudio 3 (Applied Biosystems) system. The following primer sequences were used: bovine CCN1 forward primer (Invitrogen; TTACGCTGGATGTTCGAGTG); bovine CCN1 reverse primer (Invitrogen; AACCGCATCTTCACAGTCCT); bovine glyceraldehyde 3-phosphate dehydrogenase (GAPDH; housekeeping gene) forward primer (Invitrogen; GGGTCATCATCTCTGCACCT); bovine GAPDH reverse primer (Invitrogen; GGAGGCATTGCTGACAATCT).

Zymography

Matrix metalloproteinase 2 and 9 activities were determined via zymography. Conditioned medium from (5µL or 15µL) luteal cell cultures was mixed with sample buffer [0.5M (3x) or 0.25M (2x) Tris Base, sodium dodecyl sulfate (SDS) (0.1g/mL), sucrose (80mg/mL), bromophenol blue (2mg/mL)] and loaded into a 10% acrylamide SDS separating gel containing 0.5 mg/mL gelatin. After electrophoresis at 200 volts, the gels were rinsed twice (15 minutes per wash) with 2.5% Triton X-100. The gels were then incubated in substrate buffer (0.05M Tris-HCl, 5mM $CaCl_2$, 0.05M NaCl, pH 8.0) for 24 hours at 37°C. Following an overnight incubation, the gels were stained with 0.1% Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories) for 30 minutes on a rotary shaker, followed by washing three (15 minutes per wash) times with destaining solution (10% acetic acid, 30% ethanol, 60% ddH_2O). The MMP activity was identified as clear bands against a blue background. The bands were captured using a UVP BioDoc-It^2 Imager (Analytikjena), and then digitized and quantified using UN-SCAN-IT 6.0 software.

Immunocytochemistry

Luteal cells were cultured and treated as previously described in 6-well plates containing acid treated coverslips (0.1N HCl). Preceding and following each step, cells were washed with 1X phosphate buffered saline (PBS) (1mL; 3 washes; 5 minutes). Cells were fixed with 0.4% paraformaldehyde (10 minutes), and permeabilized with 0.1% Triton X-100 in 1X PBS (15

minutes) to allow for penetration of antibodies. Non-specific binding sites were blocked with Odyssey Blocking Buffer (2 hours), before incubating the slides in the presence of rabbit anti-CCN1 antibody (1:50; Abcam) and mouse anti-CYP11A1 antibody (1:1000 or 1:500; Abcam) for 24 hours at 4°C. Following sequential incubations for30 minutes, at 37°C, with goat anti-rabbit (AlexaFlour 488; Invitrogen) and goat anti-mouse (AlexaFlour 596; Life Technologies) secondary antibodies, the cells were incubated in To-PRO-3 (30 minutes) or mounted using Prolong Gold Antifade Reagent with DAPI (Invitrogen) to counterstain the nuclei. CCN1 and CYP11A1 staining was visualized using the LSM 510 Meta laser scanning confocal microscope [Zeiss; funded by a National Science Foundation Major Research Instrumentation (MRI) grant (#0618719)].

Results

Determining CCN1 Expression by qPCR analysis

CCN1 mRNA expression in IGF-1 treated luteal cells was analyzed by qPCR and the results for each stage of CL development (early, mid, and late) are shown in

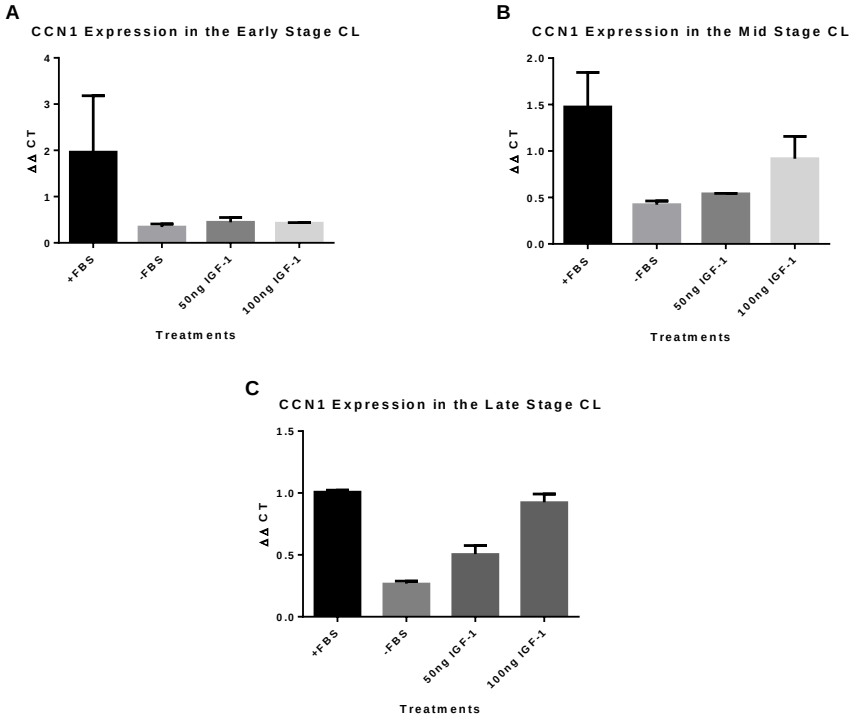

Figure **2** and 3. In the early stage CL (day 4; n=1), both concentrations (50ng/mL and 100ng/mL) of IGF-1 did not appear to affect CCN1 mRNA expression, relative to the negative control (-FBS). In the mid (day 8; n=2) and late (day 16; n=2) stage CLs, both concentrations of IGF-1 appeared to increase the expression of CCN1 relative to the negative control. However, the increase appeared greater in the late stage CL than the mid stage. In both mid and late stage CLs, the higher IGF-1 concentration appeared to induce a larger increase in CCN1 expression. Since statistics were not run on these data sets due to an insufficient number of replicates (n=1; n=2), conclusions cannot be drawn at the present time regarding the effect of IGF-1 on CCN1 expression, as well as differences in CCN1 expression between the three developmental stages of.

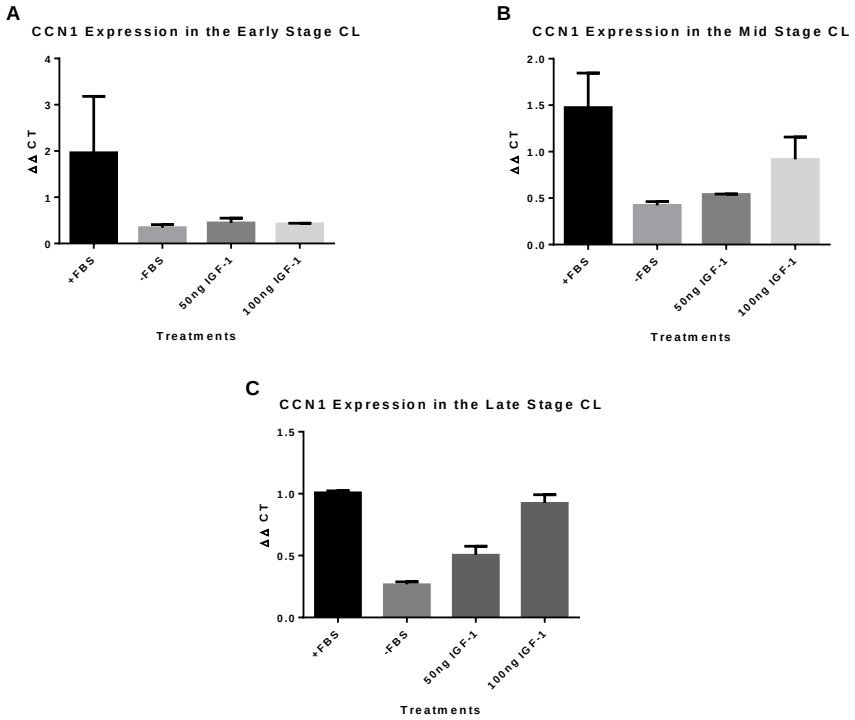

Figure 2*:* Expression of CCN1 messenger RNA (mRNA) in luteal cells from **A)** early (day 4; n=1), **B)** mid (day 8; n=2), and **C)** late (day 16; n=2) stage CLs. Luteal cells were treated with 50ng/mL or 100ng/mL of IGF-1. Control groups are +FBS and –FBS. Values are expressed as the mean ± standard deviation.

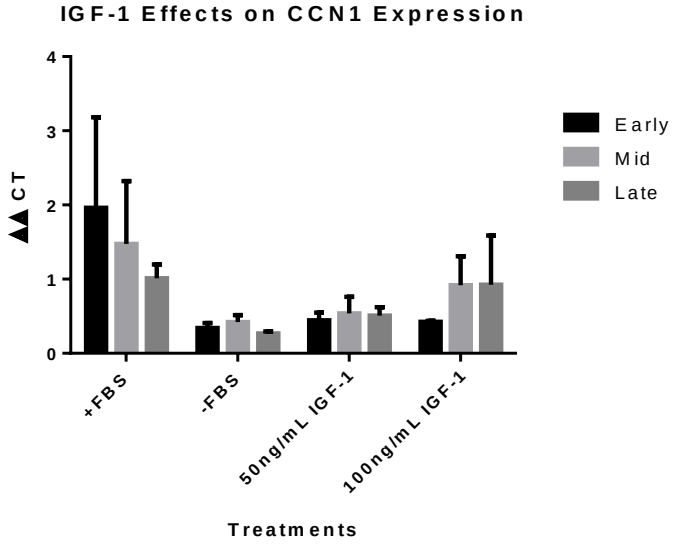

Figure 3*:* Expression of CCN1 messenger RNA (mRNA) in luteal cells from early (day 4; n=1), mid (day 8; n=2), and late (day 16; n=2) stage CLs. Luteal cells were treated with 50ng/mL or 100ng/mL of IGF-1. Control groups are +FBS and –FBS. Values are expressed as the mean ± standard deviation.

Identification of MMP-2 and MMP-9 by zymography

The expression of MMP-2 and MMP-9 in IGF-1 treated luteal cells was determined with zymography (

Figure **4**-7). On the zymograms (

Figure **4**-6; panels E and F), the MMP-2 bands are labeled 72kDa and the MMP-9 bands are labeled 92kDa.

In the early (day 4; n=1) stage CL, expression of MMP-2 (Figures 4 A and B) and MMP-9 (

Figure **4** C and D) was not affected by either concentration of IGF-1. In the mid (day 8; n=2) stage CL, the expression of both MMP-2 and MMP-9 appeared to be increased by the lower concentration of IGF-1 (Figure 5 A-D). In contrast, the addition of 100ng/mL IGF-1 had no effect on the expression of either enzyme. In the late (day 16; n=2) stage CL, expression of

MMP-2 (Figure 6A and B) and MMP-9 (Figure 6 C and D) was not affected by either concentration of IGF-1.The summary results of MMP expression by early, mid and late stage luteal cells are summarized in Figure 7.

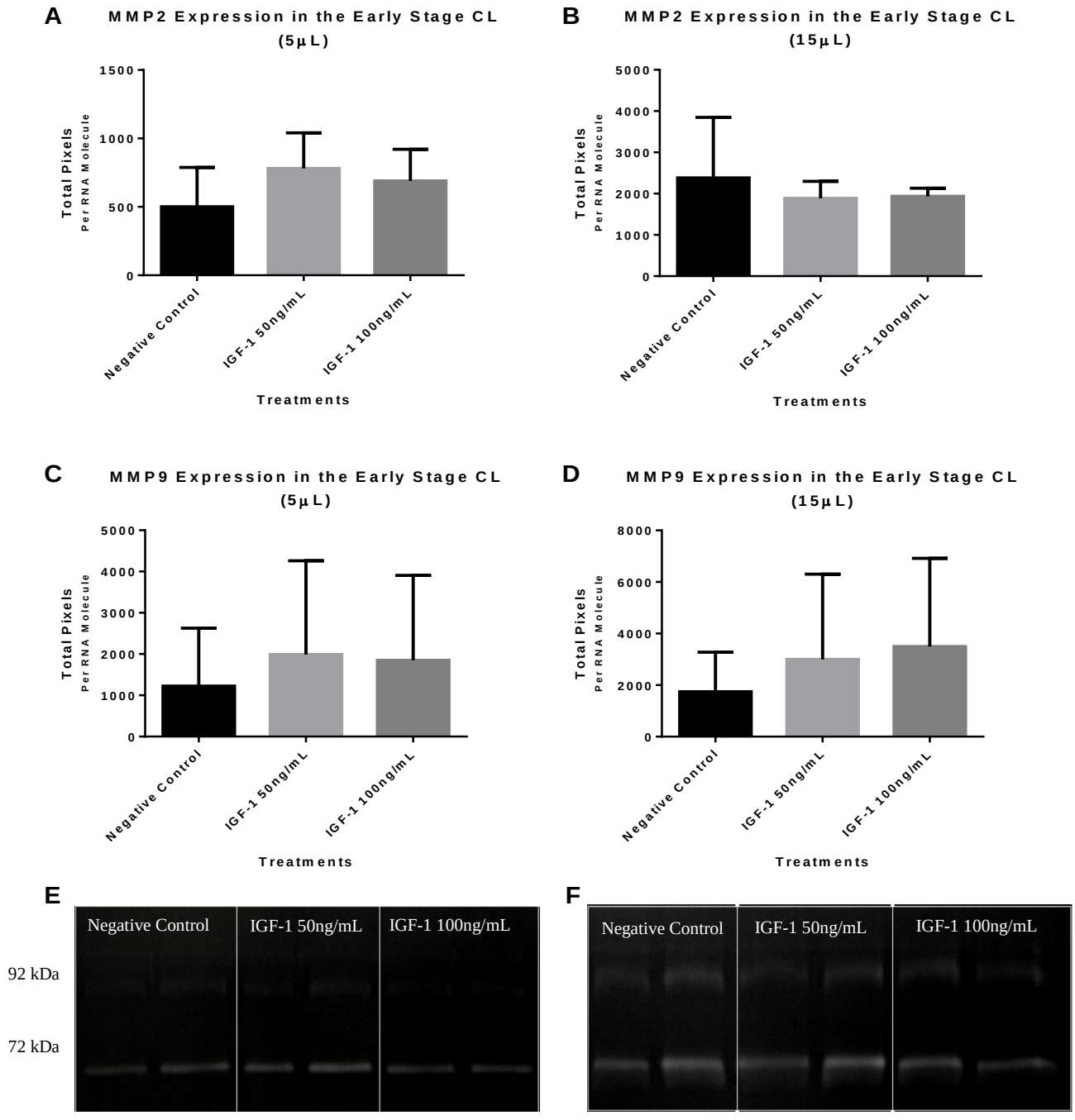

Figure 4: Expression of MMP-2 (A and B) and MMP-9 (C and D) in conditioned medium from luteal cells of an early (day 4) stage CL. Luteal cells were treated with IGF-1 (50ng/mL or 100ng/mL) for two

hours. Gels were loaded with 5 µL (A, C and E) or 15µL (B, D and F) of conditioned medium. Negative controls contained no IGF-1 or FBS. Representative gel images (E, F) show the MMP-9 band at 92 kDa and the MMP-2 band at 72 kDa. Values are expressed as the mean ± standard deviation (n=1).

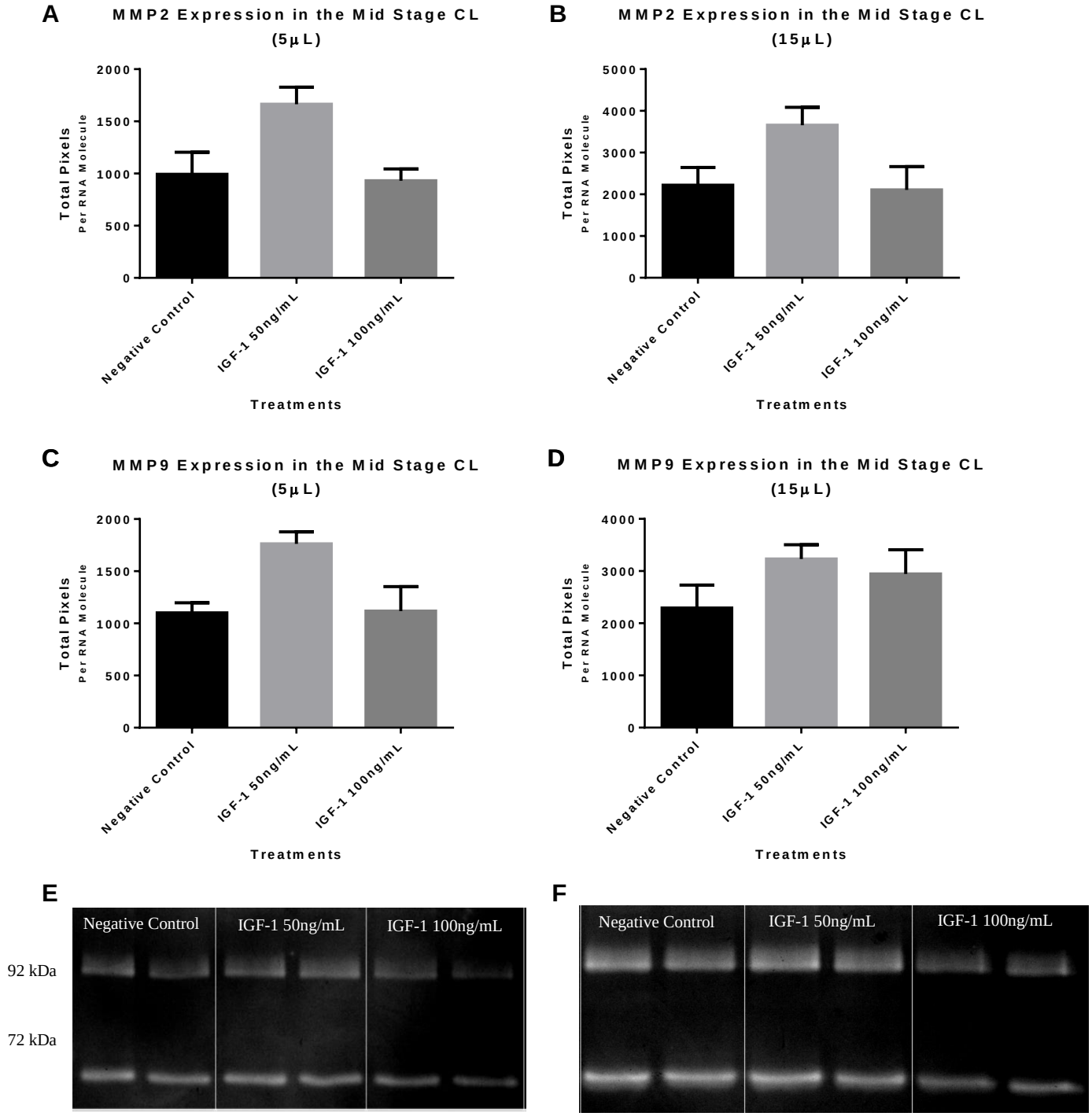

Figure 5: Expression of MMP-2 (A and B) and MMP-9 (C and D) in conditioned medium from luteal cells of mid (day 8) stage CLs. Luteal cells were treated with IGF-1 (50ng/mL or 100ng/mL) for two hours. Gels were loaded with 5 µL (A, C and E) or 15µL (B, D and F) of conditioned medium. Negative

controls contained no IGF-1 or FBS. Representative gel images (E, F) show the MMP-9 band at 92 kDa and the MMP-2 band at 72 kDa. Values are expressed as the mean ± standard deviation (n=2).

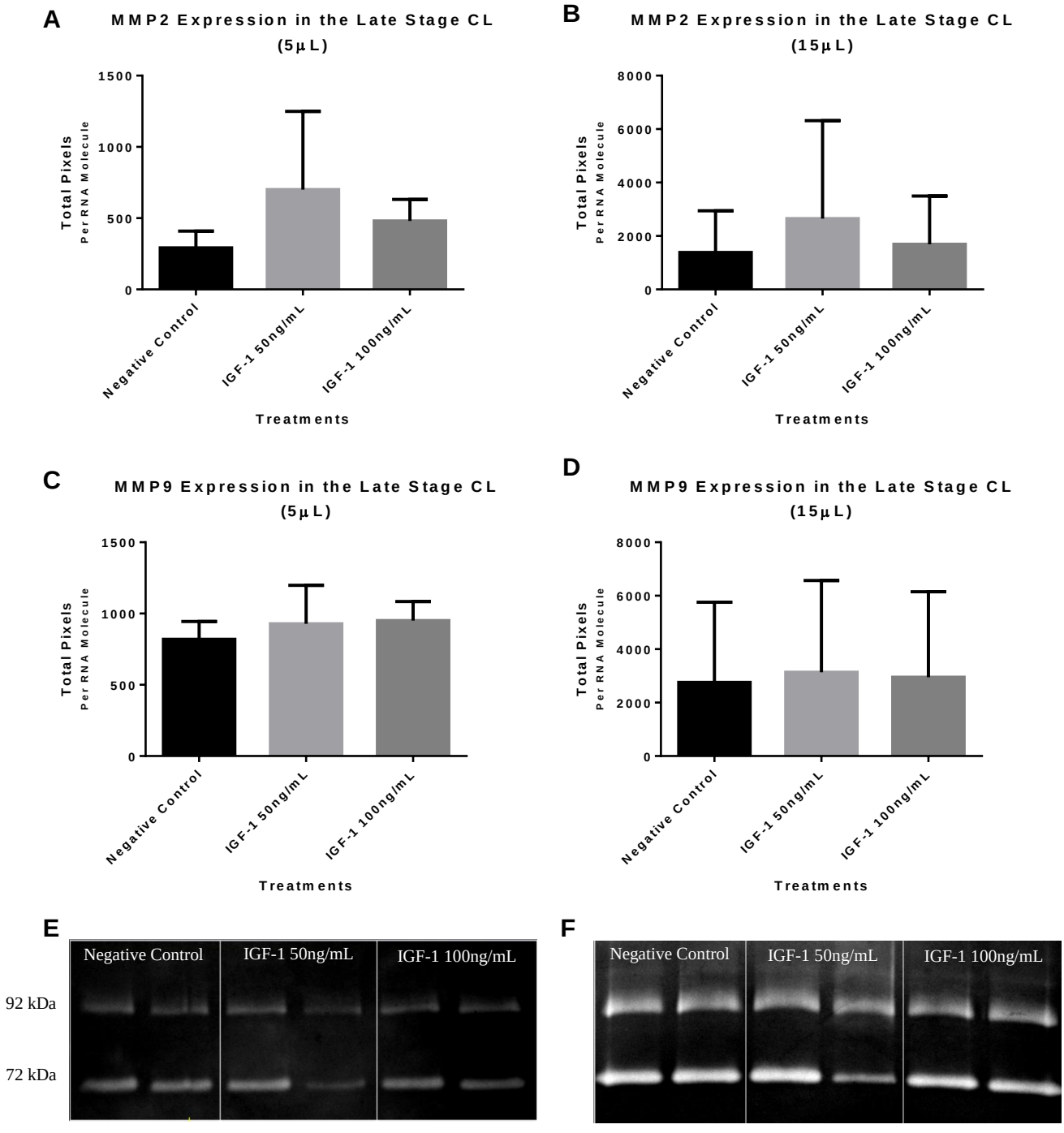

Figure 6: Expression of MMP-2 (A and B) and MMP-9 (C and D) in conditioned medium from luteal cells of late (day 16) stage CLs. Luteal cells were treated with IGF-1 (50ng/mL or 100ng/mL) for two hours. Gels were loaded with 5 µL (A, C and E) or 15µL (B, D and F) of conditioned medium. Negative

controls contained no IGF-1 or FBS. Representative gel images (E, F) show the MMP-9 band at 92 kDa and the MMP-2 band at 72 kDa. Values are expressed as the mean ± standard deviation (n=2).

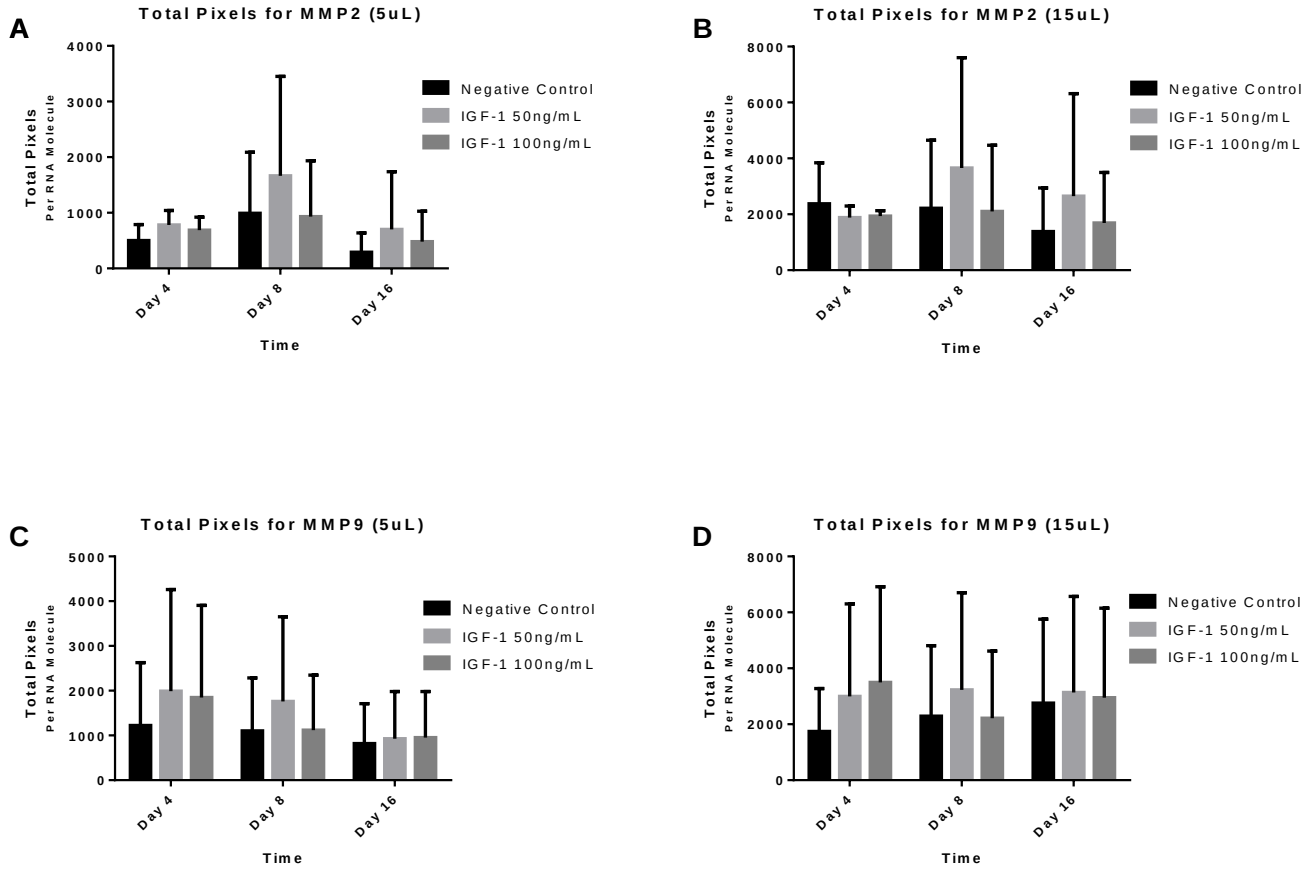

Figure 7*:* Expression of MMP-2 (A and B) and MMP-9 (C and D) in conditioned medium from luteal cells of an early (day 4; n=1), mid (day 8; n=2), and late (day 16; n=2) stage CLs. Luteal cells were treated with IGF-1 (50ng/mL or 100ng/mL) for two hours. Gels were loaded with 5 µL (A, C and E) or 15µL (B, D and F) of conditioned medium. Negative controls contained no IGF-1 or FBS. Representative gel images (E, F) show the MMP-9 band at 92 kDa and the MMP-2 band at 72 kDa. Values are expressed as the mean ± standard deviation.

Co-localization of CCN1 and CYP11A1 in bovine luteal cells

Localization of CCN1 and CYP11A1 in IGF-1 treated luteal cells obtained from early (Figure 8), mid (Figure 9) and late (Figure 10) stage CLs was determined using fluorescence immunocytochemistry. Dual staining with CCN1 (green) and the rate-limiting steroidogenic enzyme, CYP11A1 (red), revealed that the steroidogenic luteal cells are a source of CCN1.

The early (day 4) stage luteal cells treated with IGF-1 (50ng/mL or 100ng/mL) were permeabilized with methanol, not paraformaldehyde. This treatment resulted in the destruction of the integrity of the luteal cell. Due to this procedural error, no conclusions can be drawn from these images. However, for the mid (day 8) and late (day 16) stage luteal cells permeabilized with paraformaldehyde, images were sharper. Further, the intensity of color suggested that both concentrations of IGF-1 may have stimulated expression of CCN1. Due to the qualitative nature of these results and the lack of statistical analysis on the qPCR data, the differences seen in CCN1 expression are not considered significant at the present time.

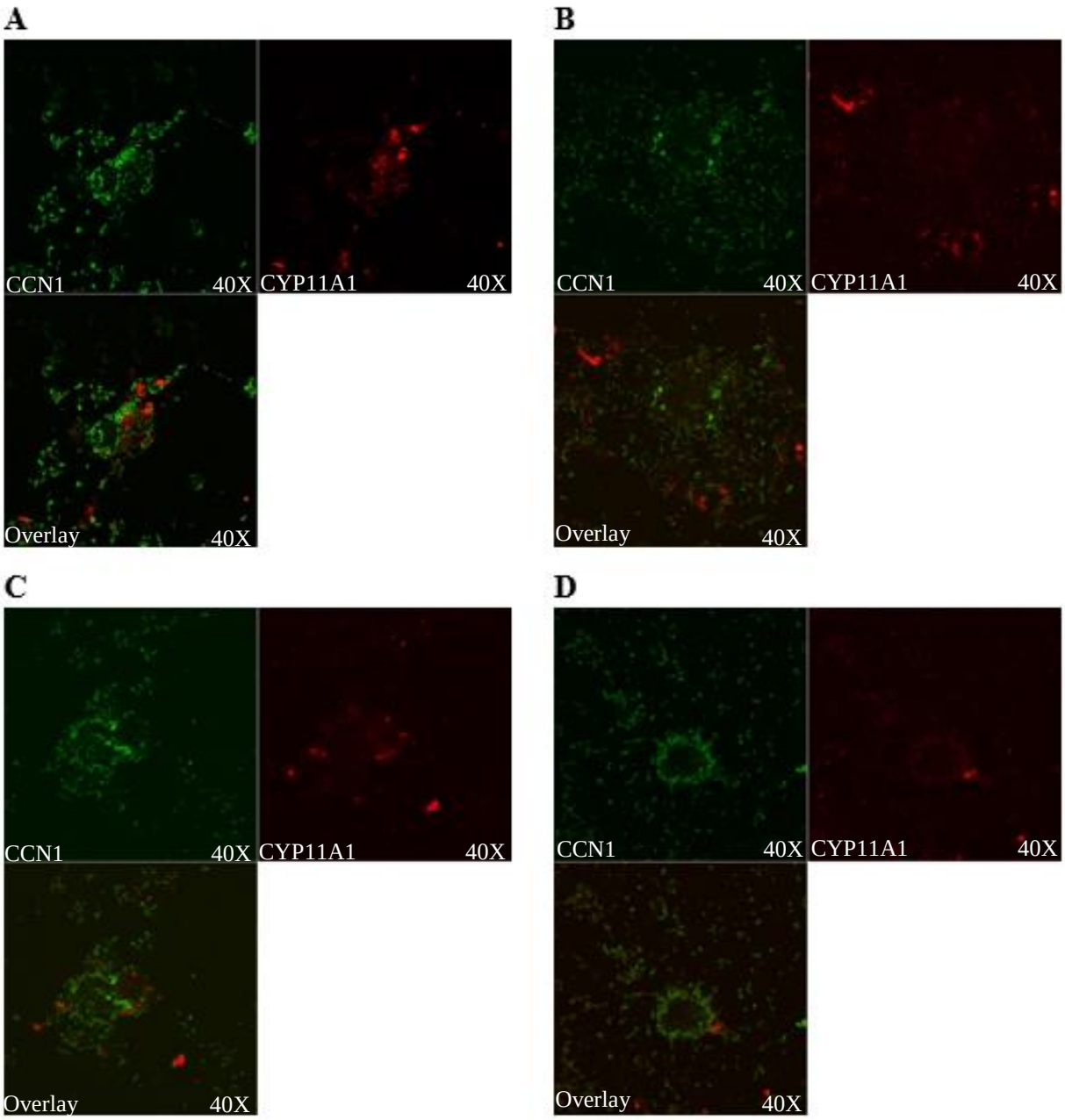

Figure 8: Immunofluorescence staining of the angiogenic inducer CCN1 (green; 1:50) and the steroidogenic enzyme CYP11A1 (red; 1:500), with image overlay (yellow) in luteal cells from an early (day 4) stage CL. Luteal cells were treated with IGF-1 at 50ng/mL (C) or 100ng/mL (D) for two hours. Negative controls contained no IGF-1 or FBS (A) and positive controls contained 10% FBS (B). Nuclear staining not shown. Magnified using a 40X oil immersion lens.

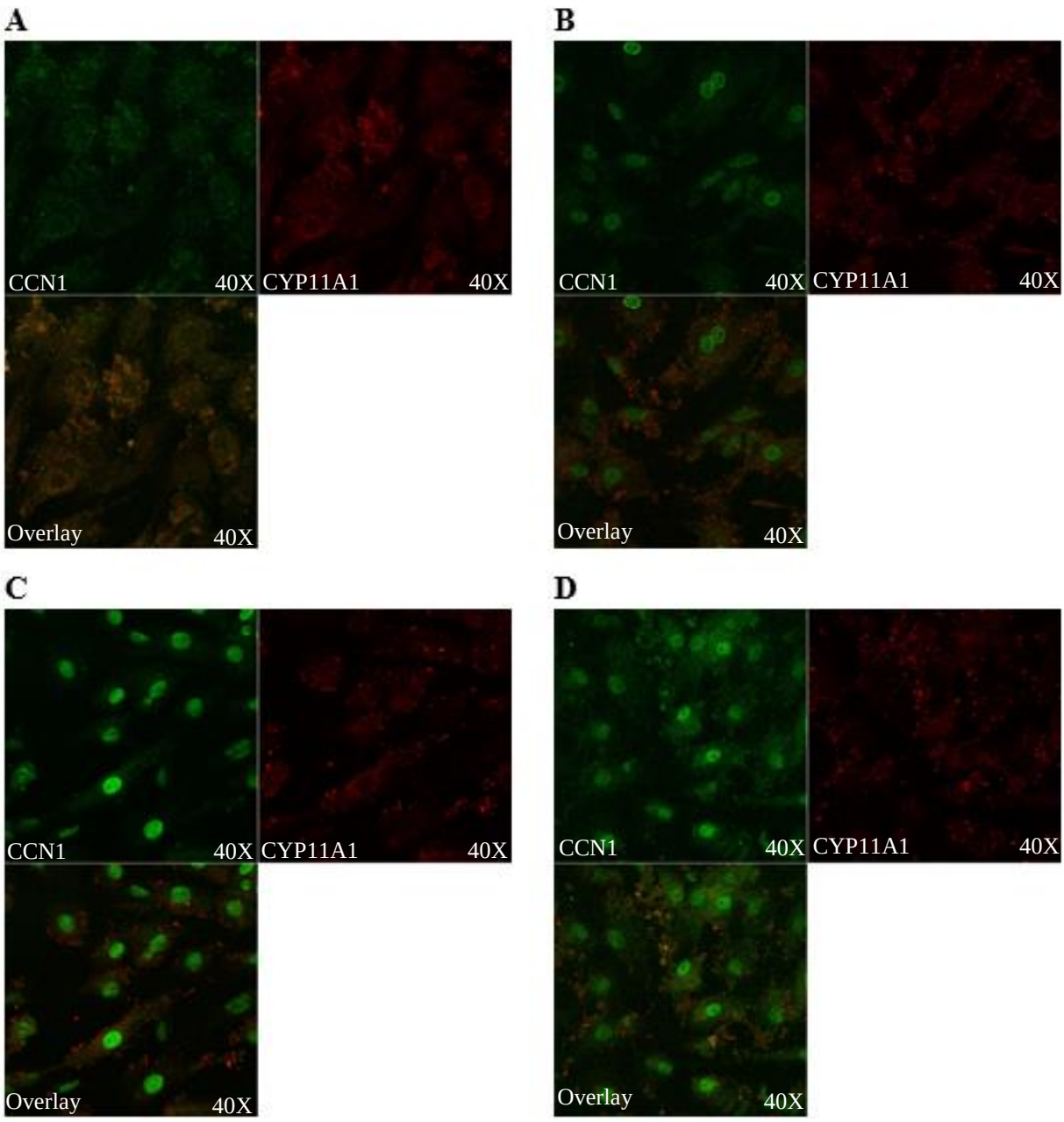

Figure 9: Immunofluorescence staining of the angiogenic inducer CCN1 (green; 1:50) and the steroidogenic enzyme CYP11A1 (red; 1:500), with image overlay (yellow) in luteal cells from a mid (day 8) stage CL. Luteal cells were treated with IGF-1 at 50ng/mL (C) or 100ng/mL (D) for two hours. Negative controls contained no IGF-1 or FBS (A) and positive controls contained 10% FBS (B). Nuclear staining not shown. Magnified using a 40X oil immersion lens.

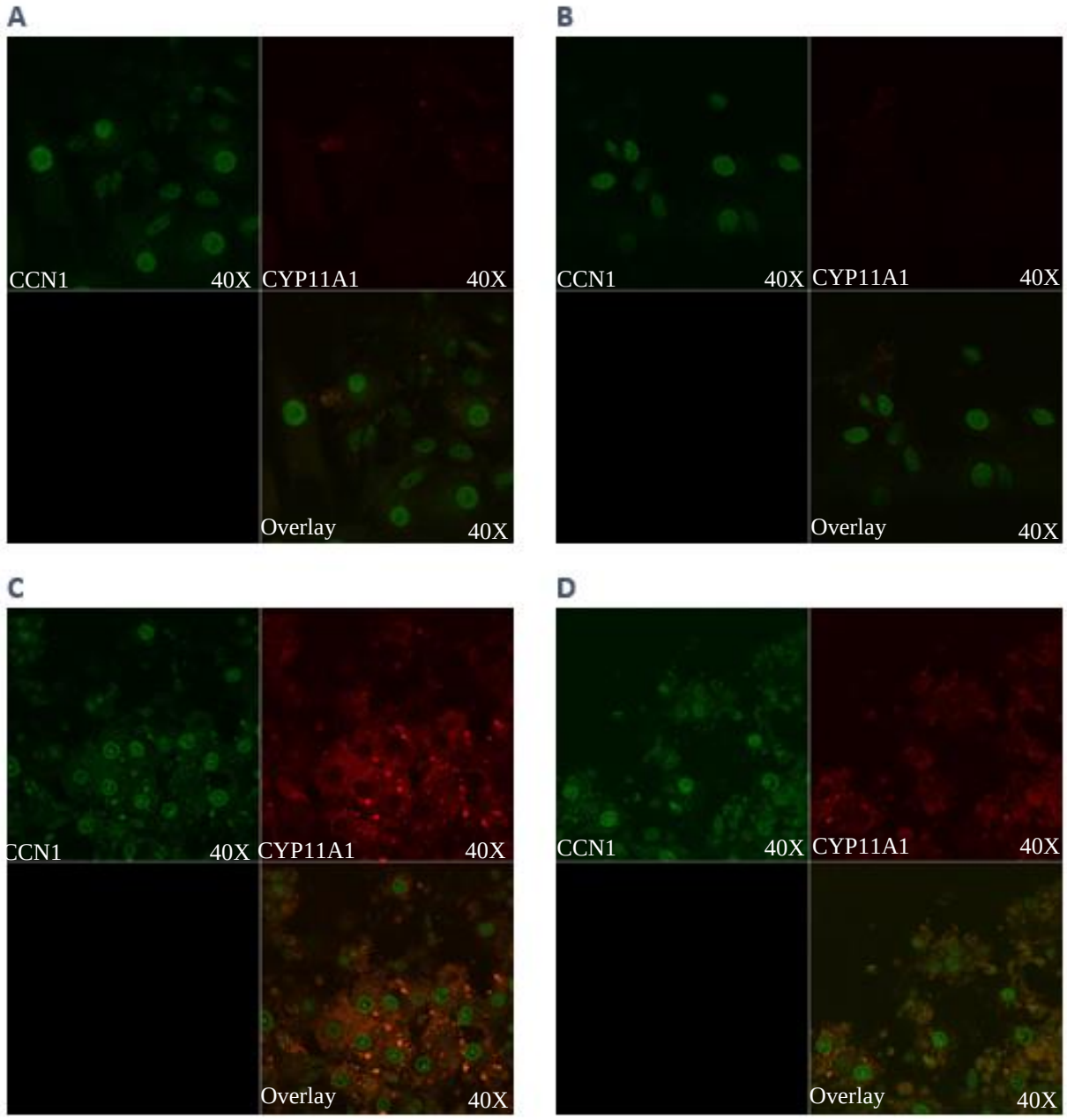

Figure 10 : Immunofluorescence staining of the angiogenic inducer CCN1 (green; 1:50) and the steroidogenic enzyme CYP11A1 (red; 1:500), with image overlay (yellow) in luteal cells from a late (day 16) stage CL. Luteal cells were treated with IGF-1 at 50ng/mL (C) or 100ng/mL (D) for two hours. Negative controls contained no IGF-1 or FBS (A) and positive controls contained 10% FBS (B). Nuclear staining not shown. Magnified using a 40X oil immersion lens.

Discussion

The present study investigated the effects of IGF-1 on CCN1 and MMP gelatinase expression in cells obtained from early (day 4; n=1), mid (day 8; n=2), and late (day 16; n=2) stage CLs. Given the need to increase the number of replicates for each stage of CL in order to perform statistical analysis, our observations of the findings so far are, at best, preliminary.

In a concurrent study from our lab group (personal communication), steroidogenic luteal cells from early, mid and late stage CLs were treated over time (2, 4, 8, and 24 hours) with FBS. The administration of FBS increased expression of CCN1 mRNA starting at 2 hours and peaked between 2 and 4 hours. In another study by Sakamoto et al. (2004), it was shown that FBS increased expression of CCN1 within 1 hour (25). Based on the results from these studies, it was determined that FBS rapidly induces CCN1 expression between 1-4 hours. Therefore, we chose a treatment time of 2 hours, which is in the middle of this range.

Several studies have looked at the role of IGF-1 in the ovary (14) (15) and its function in angiogenesis (16). These studies have found that IGF-1 has a major role in follicular development, progesterone production, and is capable of upregulating CCN1 in breast cancer cells. However, to our knowledge, this is the first study to look at IGF-1 regulation of CCN1 in the bovine CL at the early, mid, and late stages of development. In this preliminary study, we showed that both doses of IGF-1 (50ng/mL and 100ng/mL) may upregulate CCN1 mRNA expression in the mid and late stage CLs (Figure 3), a surprising finding.

The expression of CCN1 was hypothesized to be increased in the early, day 4 stage of CL development, when the rate of angiogenesis is at its peak (11). However, the role of CCN1 in the bovine CL may not be solely angiogenic; CCN1 may also play a role in apoptosis of fibroblasts (26), of which there are many in the CL. It is currently thought that CCN1 induces apoptosis via

two pathways. The first is that CCN1 binds to the integrins, αvβ5 and α6β1, and heparan sulfate proteoglycan (HSPG)-4, on the cell surface of fibroblasts, unmasking the cytotoxicity of tumor necrosis factor (TNF)-α and inducing increased levels of reactive oxygen species (ROS). In turn, the ROS activates Jun N-terminal kinases (JNK) and prepares the fibroblast for apoptosis (17). The second is that CCN1 binds to α6β1 and syndecan-4, which leads to apoptosis via the p53-independent activation of Bax and cytochrome c release (26).

The upregulation of CCN1 by IGF-1 in mid and late stage CLs was corroborated by the immunofluorescence results. While further experimentation is needed to verify our immunofluorescence results in all three stages of CL development (especially the early stage), visual observations revealed that both concentrations of IGF-1 appeared to have a positive effect on CCN1 expression in the steroidogenic luteal cells from the mid and late stage CLs. The co-localization of CCN1 and the enzyme CYP11A1 showed that the steroidogenic luteal cells are sources of CCN1.

The major protein of interest in the present study, CCN1, is not the only angiogenic factor found in the bovine CL. We also examined the effect of IGF-1 on the angiogenic regulators, MMP-2 and MMP-9. Our preliminary findings showed that IGF-1 may have differential effects on MMP-2 and MMP-9, i.e. MMP-2 is upregulated by IGF-1 in the mid and late stage CLs, while MMP-9 is upregulated mostly in the early and mid stage CLs (Figure 7).

MMPs play a role in tissue remodeling associated with many processes, including angiogenesis (20). Due to the major role of MMPs in angiogenesis, it was expected that MMP expression would be increased in the early stage of CL development when the rate of angiogenesis is at its peak (11). However, in the present study, only MMP-9 was upregulated in the early stage. Bergers et al. (2000) reported that MMP-9 was a functional component in the

angiogenic switch of pancreatic carcinomas, whereas MMP-2 was not (27). Thus, the results from this study, although in cancer and not luteal cells, may support why MMP-9, not MMP-2, was upregulated in the early stage bovine CL. Currently, we do not know why IGF-1 differentially regulates MMP expression in different stages of the bovine CL. However, Zhang et al. (2004) showed that IGF-1 upregulated MMP-2 expression in lung carcinoma cells via the pAkt and MAPK pathways (28). Conceivably, these pathways may be activated by IGF-1 in mid and late stage CLs to regulate MMP-2 expression. Thus, further study of these signaling pathways may shed light on how IGF-1 regulates not only MMPs, but also CCN1 in the bovine CL.

In summary, the preliminary findings of the present study suggest that IGF-1 play a role in CCN1 expression in later stages of CL development. Its role in the early CL awaits further study. On the other hand, IGF-1 appears to differentially regulate the two gelatinase enzymes, MMP-2 and MMP-9, depending on the stage of CL development. This, too, requires further study.

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Appendix

Luteal Cell Culture Techniques

Tissue Preparation

1. Remove excessive connective tissue from the CL
2. Weigh out the CL

   Weight = (Weight of CL + P-cup) – (Weight of P-cup)

3. Pour BSA medium into one 10mL beaker – place on ice
4. Slice CL into 1mm cubes – place into the 10mL beaker
5. Repeat step 4 until all of the CL is cleaned and cut – keep CL wet during this process
6. Using scissors, chop up the cubes in the beaker – chop for 5-10 minutes (on ice)
7. Pour the chopped tissue into the spinner flask
8. Rinse beaker 2X with BSA medium – pour into the spinner flask
9. Place spinner flask into water bath – spin on low for 1-2 minutes
10. Let tissue settle and pour liquid out
11. Add BSA medium (?) and repeat steps 9-10 two more times

Dissociation

1. Weigh out collagenase

   $$\text{Collagenase (g)} = \frac{(2000)(Weight\ of\ the\ CL)}{196} \times 0.001$$

2. Put the collagenase in a 25mL flask – add 20-25mL BSA medium
3. Add collagenase medium to the spinner flask
4. Spin for 1 hour – every 10 minutes pipet mixture up and down with the wide mouth pipet
5. After 1 hour, pour off the supernatant into two 15mL centrifuge tubes
6. Spin at 190 xG at 5°C for 10 minutes
7. Pour off supernatant – add 10mL of Ham's F12/Gen to resuspended the pellet and combine tubes
8. Repeat steps 6-7 at 110 xG and then at 80 xG
9. If needed repeat steps 1-8 for a second dissociation

Flask Preparation and Seeding

1. Add 2.5 mL of Ham's F12/Gen/Calf Serum medium to each flask/well that will be needed
2. Place flasks/plates in the incubator for 1-5 hours at 37°C
3. Before use, discard medium containing calf serum, and rinse the flasks/wells with 2.5 mL Ham's F12/Gen/ITS
4. Using the hemocytometer or cell counter, determine number of cells in the suspension

5. Determine how many cells per flask/well are needed – add the appropriate amount of suspension or dilute the suspension so you add 1mL to each flask/well

Quantitative Polymerase Chain Reaction Protocol

1. Place CCN1 and GAPDH primers (forward and reverse) and samples on ice to thaw
2. While the samples are thawing fill in the qPCR excel template and print a copy
3. Once primers have thawed, create the master mix using template calculation:
    a. CCN1:
        i. Label one RNA free tube with "MM" in red
        ii. Add water
        iii. Vortex and spin down CCN1 primers
        iv. Add forward and reverse primers
        v. Turn off the lights and add Syber-Green
        vi. Place the tube back on ice
    b. GAPDH
        i. Label one RNA free tube with "MM" in black
        ii. Repeat steps ii-vi from CCN1 preparation with GAPDH primers
4. Aliquot the master mix into the master mix plate
    a. Divide total volume of mix by 12 to determine aliquot volume
    b. Pipet the aliquot volume into the appropriate wells
    c. Place the plate back in the fridge
5. Label the fast PCR plate to demarcate CCN1 wells (red) and GAPDH wells (black)
6. Place the label plate on a cold plate
7. Place samples in the wells:
    a. Vortex and spin the first two samples
    b. Take sample one and pipette 2µL into the appropriate wells for CCN1 and GAPDH
        i. Place the pipet tip against the wall of the well
        ii. Press down to expel liquid onto side of the well
        iii. Move the pipet out of the well before releasing
    c. Repeat step B for the second sample
    d. Repeat steps A-C for the remaining samples (two samples at a time)
8. Using the multichannel pipet, add 18µL of the appropriate master mix to the appropriate wells
    a. Change tips between each addition
9. Cover the plate with the plastic film
    a. Seal cover using the tip of a P20 pipet (hold close to end of pipette)
    b. Cut off the excess plastic film with a razor blade
10. Vortex the plate for 30 seconds
11. Spin the plate down for ~1-2 minutes
12. Turn on the QuantStudio 3 machine, sign in, and select a template
13. Place plate into the machine and press "Start Run"

Zymography Protocol

Preparing the Separating Gel

1. Turn on hot plate and heat until water is boiling
2. Weigh out (0.0075mg) gelatin and pour into a 50ml centrifuge tube
3. Weigh out (0.1g) ammonium persulfate (APS) and pour into a 1.5ml tube
4. Add 7.275ml ddH_2O to the tube and heat until all gelatin has dissolved (do not boil)
5. Add 3.75ml 1.5M Tris (pH 8.8), 3.75ml 40% acrylamide, and 150ul 10% SDS to the tube
6. Add 1ml ddH_2O to 1.5ml tube containing APS and vortex until APS is completely dissolved
7. While the solution is cooling, set up gel apparatus
8. Once the solution has cooled to room temperature (26^OC = 25-40min), add 7.5uL TEMED and 75uL APS to the 50mL tube
9. Gently invert the tube to mix
10. Immediately load the separating gel solution with a Pasteur pipet between casting plates
11. Fill casting plates to the bottom of the green frame with separating gel
12. With a new Pasteur pipet, add ddH_2O until casting plates overflows
13. Allow gels to polymerize (~45min)→ use gel remaining in the 50ml tube to gauge when polymerization occurs

Preparing the Stacking Gel

1. Into a 15mL tube, add 6.36ml ddH_2O, 2.52ml 0.5M Tris (pH 6.8), 1.0ml 40% acrylamide, and 100ul 10% SDS
2. Pour off water that is layered on top of the separating gel, use blotting paper to dry
3. Add 7.5uL TEMED and 75uL APS to the 50mL tube
4. Gently invert the tube to mix
5. Immediately load the stacking gel solution with a Pasteur pipet between casting plates
6. Add the gel combs in between the plates and insure that no bubbles form

Preparing the Samples

1. Calculate the amount of sample needed to load 60ug/uL of protein
2. Based on that volume determine the amount of sample buffer that is needed
    a. 2X = 1 part buffer to 1 part protein extract
    b. 3X= 1 part buffer to 2 parts protein extract
3. Pipet the calculated amount of protein extract into a 1.5ml tube

4. Next, to the same tube, add the corresponding amount of sample buffer and aspirate to mix
5. Allow stacking gel to polymerize for ~35 min (use the remaining stacking gel to gauge when the gels are set)

Loading Samples

1. After stacking gel is set, remove glass plates from casting tower
2. Insert them into the electrode cassette with the shorter, thinner glass plate facing inward
3. Push down on the glass plates to ensure a tight fight and prevent leaking while closing the plastic flaps on clap
4. Insert cassette into running chamber
5. Remove combs
6. Pour electrode buffer into the center area between plates and allow it to over flow into the chamber
7. Using the gel loading pipet tips add marker/standard/samples according to your gel diagram

Running Gels

1. Once gels are loaded, carefully move chamber to power source
2. Attach electrode cover in proper orientation
3. Plug electrode into power source using one hand and set volts to 200 and time to 60
4. Prepare Triton X-100 (5mL Triton in 195mL of deionized water) solution
5. After dye runs off the gel, run an additional 10 min, and turn off power source

Triton Washes

1. Label the petri dish using labeling tape
2. Add ~50 mL Triton solution to labeled Petri dishes.
3. Remove glass plates and carefully extract gels, placing them in their respective dishes
4. Wash gels two times for 15 minutes (30 minutes in total) while shaking gently 49.
5. Rinse gel with ddH_2O quickly (repeat this wash 3X)
6. Fill each dish with ~150 ml of substrate buffer
7. Place on shaker in 37ºC incubator and shake gently overnight (i.e. barely oscillating)

Staining Gels

1. Add ~50 mL of Coomassie solution to each dish
2. Shake gently at room temperature for 30 minutes
3. Using a 25mL pipet to remove Coomassie from each dish and dispose in Hazard Waste bottle in hood

Destaining Gels

1. Add ~ 50 mL destaining buffer to each gel and shake at room temperature for 15min
2. Using a 25mL pipet remove destaining buffer from each dish and dispose in Hazard Waste Bottle in hood
3. Repeat this wash 2X (total of 3, 15min washes)
4. Last wash, remove destaining buffer and store gel in a little ddH_2O
5. Within 1 hour photograph the gel

Immunocytochemistry Protocol

Cell Seeding:

1. Acid wash coverslips
    a. Dip each coverslip into 0.1N HCl solution
    b. Follow with two washes in sterilized water
    c. Leave the coverslips to air dry on a Kimwipe
    d. Place coverslips in a petri dish and autoclave
2. In the hood, use ethanol-sterilized forceps to place one sterilized coverslip into each well (6 or 12 well plate)
3. Passage the hGran cells and seed the plates with 100,000 cells/well in 2mL of DMEM + 10%FBS medium
4. Incubate the cells at 37°C for 48 hours

Cell Treatments:

1. Save 2mL of the pre-treatment media in cryovial tubes
    a. Aspirate off the media
    b. Replace with 2mL of DMEM with, 1) nothing added, 2) 10% FBS, 3) 50ng/mL IGF-1, or 4) 100ng/mL IGF-1
    c. Incubate at 37°C for 2 hours
2. Add the treatments to each well (dependent of experiment)
3. Save 2mL of the post-treatment media in cryovial tubes

Cell Fixing:

1. Wash the cells two times for 5 minutes (10 minutes total) with 2mL/well of 1X PBS while gently shaking
2. Aspirate the wash medium, add 1mL of 4% paraformaldehyde in 1X PBS
3. Keep the plates at room temperature for 10 minutes
4. Aspirate fixative and add 1mL of 0.1% triton to permeabilize the cells
5. Keep the plates at room temperature for 15 minutes
6. Wash the cells again three times for 5 minutes (15 minutes total) with 2mL/well of 1X PBS while gently shaking

Blocking:

1. Set up the incubating chamber
    a. Place two wet paper towels at the bottom of the pipet tip box
    b. Place two strips of Parafilm on top of the tip rack
2. Pipette 45uL of Odyssey Blocking Buffer onto the Parafilm (total of 3 times per Parafilm strip – 6 times total)
3. Lay the coverslips, cell-side down, using forceps onto the Parafilm
4. Incubate at room temperature for 2 hours

Primary Staining:

1. Remove the coverslips using forceps, replace them to the original well plate cell-side up
2. Wash 3x for 5 minutes (15 minutes total) with 1mL/well of 1X PBS
3. Dilute primary antibodies to appropriate concentration in Odyssey Blocking Buffer
4. Pipet 40uL of primary antibody onto the Parafilm
5. Lay the coverslips, cell-side down, using forceps onto the Parafilm
6. Incubate at -4C° for 16-24 hours

Secondary Staining (Light-Sensitive):

1. Remove the coverslips using forceps, replace them to the original well plate cell-side up
2. Wash 3x for 5 minutes (15 minutes total) with 1mL/well of 1X PBS
7. Dilute secondary antibodies to appropriate concentration in Odyssey Blocking Buffer
3. Pipette 40uL of secondary onto the Parafilm
4. Lay the coverslips, cell-side down, using forceps onto the Parafilm
5. Incubate in the dark at room temperature for 1 hour, or at 37°C for 30 minutes

Mounting:

1. Remove the coverslips using forceps, replace them to the original well plate cell-side up
2. Wash 3x for 5 minutes (15 minutes total) with 1mL/well of 1X PBS in the dark
3. Pipette 8µL of Prolong Gold Antifade Reagent with DAPI onto labeled microscope slides
4. Lay the coverslips, cell-side down, using forceps onto the microscope slides
5. Allow the mounting medium to cure overnight at room temperature or in the fridge, keep the microscope slides flat

Antibody Dilutions:

Primary Antibody	Raised In	Dilution	Solution
CCN1	Rabbit	1/50	Blocking Buffer
CYP11A1	Mouse	1/500	Blocking Buffer

Secondary Antibody	Raised In	Dilution	Solution
AlexaFlour 488 Anti Rabbit	Goat	1/1000	Blocking Buffer
AlexaFlour 596 Anti Mouse	Goat	1/1000	Blocking Buffer