CHAPTER 4

PAPER III:

Collagen I Gel Substrate Increases the IGFBP-3/IGFBP-2 Ratio of Secretion for a Bovine Mammary Epithelial Cell Line

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Running Title: Collagen I Gel Increases IGFBP-3 Production

4.1 Abstract

Insulin-like growth factor-I (IGF-I) regulation by IGF binding proteins is well documented and many cells, including mammary epithelial cells, secrete measurable levels of IGFBPs. The IGF-I axis (IGF-I, IGF-I receptor (IGF-IR), and modulating IGF binding proteins (IGFBPs)) is important to mammary growth and development. In particular, IGFBP-3 has been shown to regulate the IGF-I response in the MAC-T cell line, a hormonally responsive immortalized bovine mammary epithelial cell line. Extracellular matrix (ECM) proteins have also been found to impact cell response as documented in the literature. The present study focused on whether IGF-I induced IGFBP-3 when cells were cultured on collagen I and whether the collagen I had additional effects on other IGFBP production. Both confluent and sub-confluent cells were investigated. We show that adherence of MAC-T cells on a collagen I gel increases not only the secretion of IGF-I stimulated IGFBP-3 into the media but also the basal secretion of IGFBP-3. In addition, it was found that cells plated on a collagen I gel secreted a decreased amount of IGFBP-2 into the media as compared to cells cultured on tissue culture plastic or a thin layer of collagen I. The overall result for both confluent and sub-confluent cells on a collagen I gel is an increase in the ratio of IGFBP-3 to IGFBP-2 in the conditioned media. The altered IGFBP secretion by confluent MAC-T cells plated on a collagen I gel cannot be accounted for by an increase in basal IGF-I receptor phosphorylation nor by differences in IGF-I uptake as measured by IGF-I content in conditioned media. Northern blot analysis did not reveal an increase in IGFBP-3 mRNA for confluent cells cultured on collagen I gel as compared to cells

cultured on tissue culture plastic or on a thin layer of collagen I. Cells on collagen I gel showed a decrease in IGFBP-2 mRNA for confluent cells with or without IGF-I addition. Moreover, cells cultured on the other two surfaces (tissue culture plastic and thin layer of collagen I) showed a decrease in IGFBP-2 mRNA in the presence of exogenous IGF-I incubation. Further investigations are needed to resolve the difference in message and protein levels for the IGFBPs and determining what signaling pathways are involved in mediating these collagen I gel induced phenomena.

Key Words: Collagen I ; IGFBP; insulin-like growth factor-I; MAC-T; mammary epithelial cells

4.2 Introduction

Extracellular matrix (ECM) proteins are receiving increasing attention in the literature because of the impact of these proteins on growth factor associated cell responses *(1-5)*. Components of the ECM can affect the growth and differentiation of cells, both by integrin receptor signaling *(1,2)* and by affecting the geometric shape of cells *(6)*. Of particular interest in this study is the impact of collagen I substrates on the IGF-I signaling pathway in mammary epithelial cells. Collagen I was chosen because it is a ubiquitous ECM protein associated with tissue structure and it is found in mammary tissues *(7,8)*. The MAC-T cell line was chosen because it is an immortalized bovine mammary epithelial cell line that has retained IGF-I responsiveness as demonstrated in previous studies *(9-11)*. Signaling by IGF-I via the insulin-like growth factor receptor

(IGF-IR) is initiated by binding of the ligand to the receptor *(12)*. Autophosphorylation of tyrosine kinase domains on the IGF-IR subunits follows, initiating numerous events in the signaling cascade of the IGF-IR. One of the previously reported responses of MAC-T cells to IGF-I stimulation is the increased production of IGFBP-3 *(13)*. IGFBP-3 is known to modulate the ability of IGF-I to stimulate the IGF-IR, presumably by sequestration of IGF-I by the binding protein *(14)*. The question this study sought to address was whether adherence to collagen I could impact the cell utilization of exogenous IGF-I and the secretion of IGFBPs, which might ultimately impact IGF-I mediated activity. If collagen I were to impact IGF-I pathway mediated activity, one could envision using a collagen modified scaffold or surface to alter IGF-I mediated cell response.

In this study, collagen I was used in two different substrate forms because it has been demonstrated in other studies that geometrical considerations and cell deformation can impact cell apoptotic behavior on fibronectin, an adhesive ECM protein *(6)*. It was found that the culturing of MAC-T cells on a collagen I gel substrate, but not a collagen I thin film (BIOCOAT), increased the secretion of IGFBP-3 and decreased the secretion of IGFBP-2 by cells when compared with cells on tissue culture plastic. To determine if the IGFBP-3 secretion by confluent cells resulted from activation of the IGF-IR signaling pathway, IGF-IR phosphorylation, IGF-IR quantity, and the levels of IGFBP-3 mRNA and of IGFBP-2 mRNA were determined. Measurement of IGF-I content in the media showed no difference in cell utilization by cells on the three different surfaces. Experiments to detect differences in the initial steps of the IGF-I signaling pathway

(exogenous IGF-I and IGF-IR phosphorylation) did not reveal any differences between the three substrates, suggesting that altered activation of the IGF pathway was not involved in the change in the IGFBP ratio found. Further, IGF-I stimulation of MAC-T cells on all three surfaces revealed a decrease in IGFBP-2 mRNA as measured by Northern blot, which did not correspond with a decrease in IGFBP-2 secretion into the media by the cells cultured on tissue culture plastic or on collagen I BIOCOAT surfaces. In fact, a significant reduction in secreted IGFBP-2 as measured from the media occurred only from cells cultured on the collagen I gel.

From this study, it was found that the collagen I gel impacted IGFBP-3 and IGFPB-2 secretion by cultured mammary epithelial cells into the media. The simple presence of collagen I as shown by the collagen I BIOCOAT was not enough to effect this difference, suggesting geometric presentation of the cell and/or the collagen I was important. Further studies are needed to characterize the interaction of the cell with the gel surface, including the shape of the cell on the gel surface and if cells migrated into the gel, as this was not measured nor photographed.

4.3 Results

DNA Content of Cells Comparable on All Three Surfaces

Surface substrate could impact cell attachment or growth rate which ultimately might impact the IGF-I axis. To address this, DNA analysis of cells was performed for all three experimental surfaces (tissue culture plastic, collagen I BIOCOAT, and collagen I gel) at the two different cell seeding densities (50,000 cells/well and 200,000 cells/well) after the collection of conditioned media. No differences in DNA content for cells adhered on the three different substrate surfaces at either cell seeding density were evident (Figure 4.1). Addition of IGF-I (100 ng/mL) stimulated comparable increases in DNA on all three different surfaces.

Collagen I Gel Increases IGFBP-3 Secretion and Reduces IGFBP-2 Secretion in MAC-T Cells

Collagen I has been shown to impact both IGF-I and IGFBP secretion in muscle cells *(15),* leading to the hypothesis that changes in the IGF-I axis of mammary epithelial cells can result from altered ECM attachment. To address this, MAC-T cells were cultured on tissue culture plastic, collagen I BIOCOAT (pre-coated plastic), and collagen I gel (300μL/well; 2 parts collagen stock solution to 3 parts gel). Wells were seeded with 50,000 cells/well (25,000 cells/cm^2) to represent a sub-confluent cell layer or 200,000 cells/well (100,000 cells/cm^2) to represent a confluent cell layer, thereby addressing the

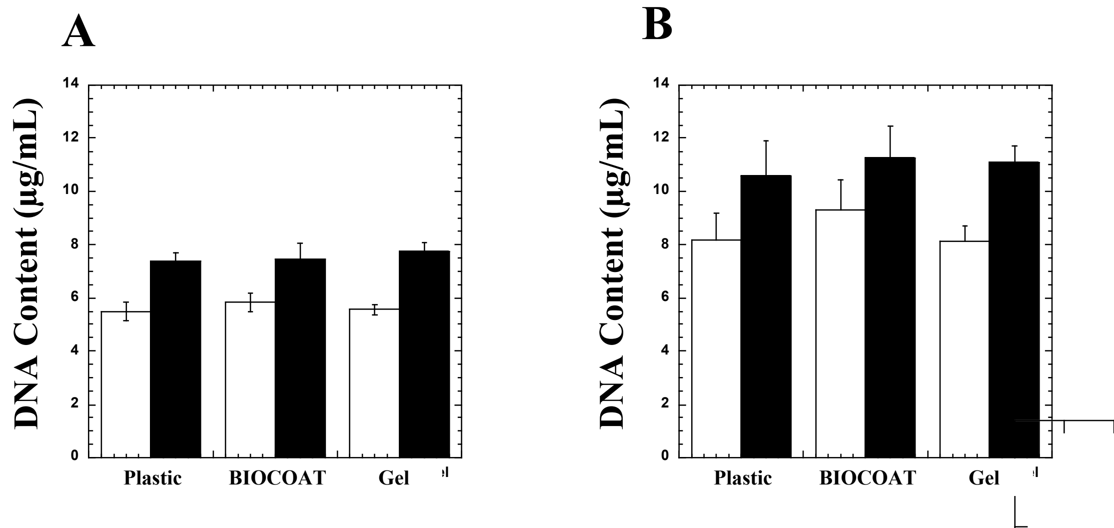


Figure 4.1. DNA content of cells seeded at **(A)** 50,000 cells/well and **(B)** 200,000 cells/well. DNA analysis of cells was conducted immediately after removal of conditioned media for ligand blot analysis. Cells were incubated either without IGF-I (open columns) or with 100ng/mL IGF-I (solid columns) on tissue culture plastic, collagen I BIOCOAT, or collagen I gel. Error bars represent standard errors of the means (pooled data from 5 independent experiments ; 4 wells were analyzed for each condition in each experiment).

effect of cell confluency on IGFBP secretion. IGFBP-3 and IGFBP-2 were detected in the media of cells on all three experimental surfaces in the presence and in the absence of IGF-1 (100 ng/mL) by ligand blot analysis (Figure 4.2). Figure 4.2 (A) shows three representative blots; blot A-1 is a shorter exposure blot to reveal IGFBP-2 bands, and blot A-2 is an over-exposed film of blot A-1 to further bring out the IGFBP-3 bands. Blot A-3 is from a separate experiment to show the reproducibility of Blot A-2. For both seeding densities, bands corresponding only to the molecular weights of IGFBP-3 and IGFBP-2 were found. Note that [^{125}I]IGF-I was used as the probe and not IGF-II, making detection of IGFBP-6 difficult *(16)*. IGFBP-2 levels were greater than those of IGFBP-3 on all three surfaces. The addition of IGF-I (100ng/mL) resulted in an increase in IGFBP-3

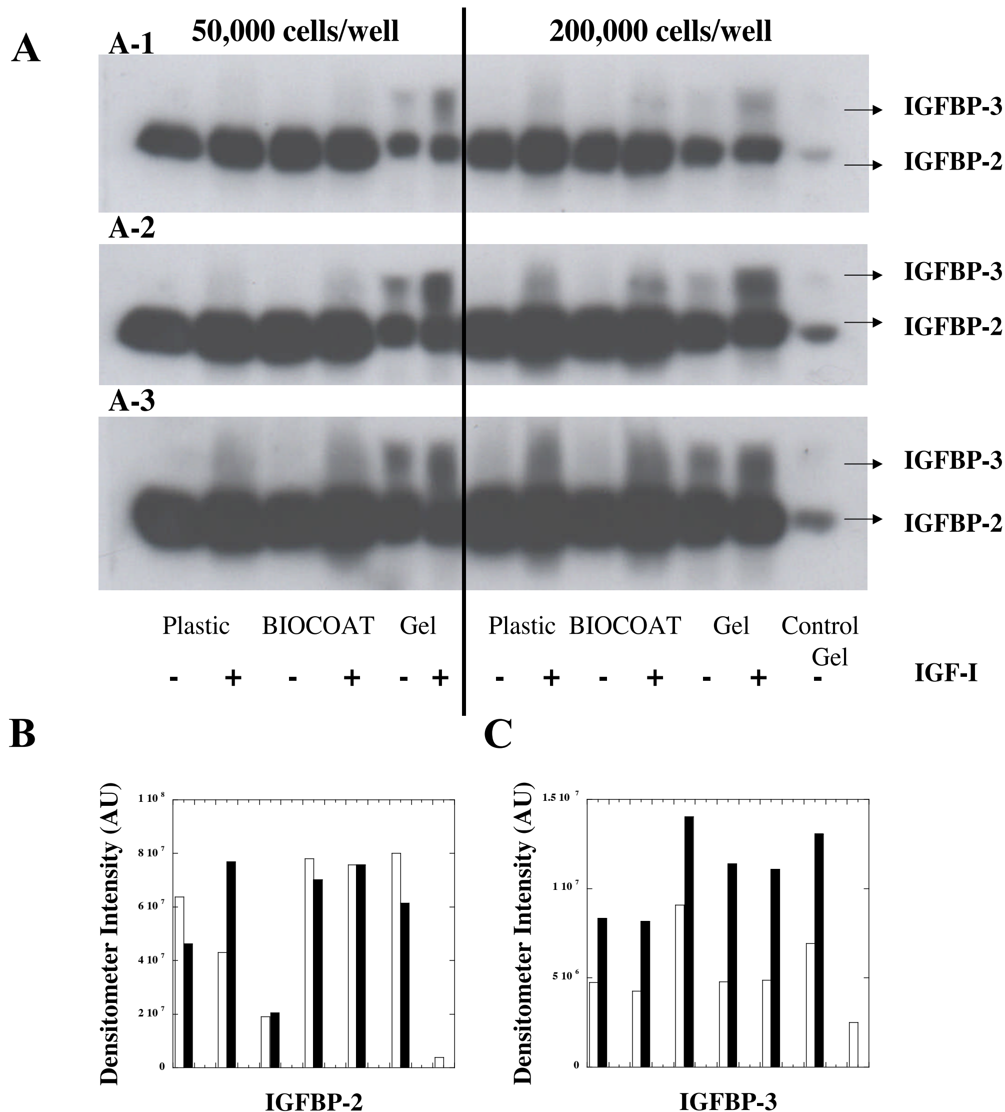


Figure 4.2. (A) Representative ligand blots showing IGFBP-3 and IGFBP-2 bands. Media for ligand band detection were collected from wells seeded with 50,000 cells/well and 200,000 cells/well and incubated 48 h with or without 100 ng/mL IGF-I on the three different surfaces: tissue culture plastic, collagen I thin coat (BIOCOAT), and collagen I gel. Blot **A-1** was exposed to detect IGFBP-2 levels. Blot **A-2** is a longer exposure of blot **A-1,** which overexposed the IGFBP-2 bands as seen, but allowed IGFBP-3 to be seen more clearly. Blot **A-3** is from a separate experiment to show repeatability of results. Densitometer readings of the IGFBP-2 **(B)** and IGFBP-3 **(C)** band intensities are shown in the column graphs. The columns are in the same order from left to right as the order in which the bands appear on the blots. Collagen I gel cell-free control band intensities have been subtracted from the collagen I gel results, but are shown for comparison. Open columns are for lanes without IGF-I; solid columns are for lanes with IGF-I (100 ng/mL). This data is representative of five independent experiments, two of which were configured with data on a single blot as shown.

band intensity from the media from cells cultured on all three surfaces. Figure 4.2(B) shows the band intensities for IGFBP-2 using densitometry software. From this graph, a decrease in IGFBP-2 secretion by MAC-T cells at sub-confluence on the collagen I gel is observed. Figure 4.2(C) illustrates the increase in IGFBP-3 band intensity with IGF-I addition by densitometry. With regard to surface effects, bands for IGFBP-3 are clearly more intense for the collagen I gel compared to the tissue culture plastic and collagen I BIOCOAT.

Densitometry measurements of the IGFBP-3 and IGFBP-2 bands were read from blots from five separate experiments with the goal of pooling the measurements and of determining a quantitative measure of comparison of IGFBP secretion by cells cultured on the three different surfaces. Although the increase in IGFBP-3 band intensities could be visualized from blot to blot, the quantification proved disappointing in that there was too much variability in the densitometry measurements, and the significance could not be captured using the densitometry method. This is discussed in detail in Chapter 5.

Table 4.1 and Table 4.2 show the visualized results from three of the five experiments (one experiment did not have data for 200,000 cells). The combined densitometry data results from the ligand blots from two separate experiments with complete data sets are shown in Chapter 5 (Figure 5.2.) Although a total of five separate experiments were performed, two of these experiments did not have lanes for both 0 and 100 ng/mL IGF-I on the same ligand blot. The three experiments for which results are shown in Tables 4.1 and 4.2 each had a single blot with lanes for both 0 and 100 ng/mL IGF-I on the same

blot. Tables 4.3. and 4.4 show the data from the other two separate experiments. For these other two experiments, the lanes for 0 and 100 ng/mL IGF-I were on different blots, and each reported intensity is from a separate blot, representing four replicate lanes on that blot. The overall results, taking into consideration all of the data at both seeding levels, are that IGFBP-3 is increased in the media of cells cultured on a collagen I gel and that IGFBP-2 is decreased in the media of cells cultured on a collagen I gel, with or without added IGF-I (100 ng/mL).

Increase in IGFBP-3/IGFBP-2 Ratio of Secretion Increased with Collagen I Gel

It is intuitive that the IGFBP-3/IGFBP-2 ratio of secretion on a collagen I gel is increased from the results of the previous section where the overall results show an increase in IGFBP-3 secretion and a decrease in IGFBP-2 secretion. Although densitometry data revealed a decrease in IGFBP-2 secretion from cells on a collagen I gel, the variability in densitometry measurements precluded conclusions on IGFBP-3 secretion based on those measurements alone. Densitometry results are shown and discussed in Chapter 5 (Figure 5.2)

TABLE 4.1

Relative intensity of IGFBP-3 bands on three separate blots from three independent experiments at 50,000 cells/well and at 200,000 cells/well. Blots were made in duplicate for each experiment to verify results. (Duplicate data not reported; intensity is reported once and represents both results.)

50,000 cells/well

Blot 1:	**IGF-I Added**	**Plastic**	**Collagen I BIOCOAT**	**Collagen I Gel**
	100 ng/mL	+	+	++
	0 ng/mL	0	0	+

Blot 2:	**IGF-I Added**	**Plastic**	**Collagen I BIOCOAT**	**Collagen I Gel**
	100 ng/mL	+	+	++
	0 ng/mL	0	0	++

Blot3:	**IGF-I Added**	**Plastic**	**Collagen I BIOCOAT**	**Collagen I Gel**
	100 ng/mL	+	+	++
	0 ng/mL	0	0	++

200,000 cells/well

Blot 1:	**IGF-I Added**	**Plastic**	**Collagen I BIOCOAT**	**Collagen I Gel**
	100 ng/mL	++	++	++
	0 ng/mL	0	0	++

Blot 2:	**IGF-I Added**	**Plastic**	**Collagen I BIOCOAT**	**Collagen I Gel**
	100 ng/mL	+	+	++
	0 ng/mL	0	0	+

Relative intensities are determined as follows:

0 no discernible band
+ faint band
++ distinct, strong band
+++ overexposed band

TABLE 4.2

Relative intensity of IGFBP-2 bands on three separate blots from three independent experiments at 50,000 cells/well and at 200,000 cells/well. Blots were made in duplicate for each experiment to verify results. (Duplicate data not reported; intensity is reported once and represents both results.)

50,000 cells/well

Blot1:	**IGF-I Added**	**Plastic**	**Collagen I BIOCOAT**	**Collagen I Gel**
	100 ng/mL	+++	+++	++
	0 ng/mL	+++	+++	++

Blot 2:	**IGF-I Added**	**Plastic**	**Collagen I BIOCOAT**	**Collagen I Gel**
	100 ng/mL	+++	+++	++
	0 ng/mL	+++	+++	++

Blot 3:	**IGF-I Added**	**Plastic**	**Collagen I BIOCOAT**	**Collagen I Gel**
	100 ng/mL	+++	++	++
	0 ng/mL	+++	++	+++

200,000 cells/well

Blot 1:	**IGF-I Added**	**Plastic**	**Collagen I BIOCOAT**	**Collagen I Gel**
	100 ng/mL	+++	+++	++
	0 ng/mL	+++	+++	++

Blot 2:	**IGF-I Added**	**Plastic**	**Collagen I BIOCOAT**	**Collagen I Gel**
	100 ng/mL	+++	+++	++
	0 ng/mL	+++	+++	++

Relative intensities are determined as follows:

0 no discernible band
+ faint band
++ distinct, strong band
+++ overexposed band

TABLE 4.3

Relative intensity of IGFBP-3 bands on separate blots from two independent experiments at 50,000 cells/well and at 200,000 cells/well. (Each relative intensity reported represents the intensity of four replicates on the same blot.)

50,000 cells/well

IGF-I Added	Plastic	Collagen I BIOCOAT	Collagen I Gel
100 ng/mL	0 [4] 0 [5]	0 to + (diffuse band) [4] 0 [5]	+++ [4]
0 ng/mL	0 [4] 0 [5]	0 [4]	+ [4]

200,000 cells/well

IGF-I Added	Plastic	Collagen I BIOCOAT	Collagen I Gel
100 ng/mL	+ [4] 0 [5]	++ [5]	+++ [4] ++ [5]
0 ng/mL	0 [5]	+ [4] 0 [5]	++ [4] ++ [5]

Relative intensities are determined as follows:
0 no discernible band
+ faint band
++ distinct, strong band
+++ overexposed band

Each reported intensity represents four replicate lanes on an individual blot:
[4] Individual blots made from media from experiment 4
[5] Individual blots made from media from experiment 5

TABLE 4.4

Relative intensity of IGFBP-2 bands on separate blots from two independent experiments at 50,000 cells/well and at 200,000 cells/well. (Each relative intensity reported represents the intensity of four replicates on the same blot)

50,000 cells/well

IGF-I Added	Plastic	Collagen I BIOCOAT	Collagen I Gel
100 ng/mL	+ [4] + [5]	+++ [4] +++ [5]	+++ [4]
0 ng/mL	+ [4] + [5]	+++ [4]	++ [4]

200,000 cells/well

IGF-I Added	Plastic	Collagen I BIOCOAT	Collagen I Gel
100 ng/mL	+++ [4] +++ [5]	+++ [5]	+++ [4] +++ [5]
0 ng/mL	+++ [5]	+++ [4] +++ [5]	++ [4] ++ to +++ [5]

Relative intensities are determined as follows:
0 no discernible band
+ faint band
++ distinct, strong band
+++ overexposed band

Each reported intensity represents four replicate lanes on an individual blot:
[4] Individual blots made from media from experiment 4
[5] Individual blots made from media from experiment 5

No Change in Signaling Pathway Detected in Selected Assays

Given that we observed an increase in IGFBP-3 in the media of cells cultured on collagen I gel above that of cells cultured on tissue culture plastic or collagen I BIOCOAT (Tables 4.1-4.4), we investigated whether plating cells on collagen I gel might result in either a change in consumption of IGF-I or increased phosphorylation of IGF-IR, thereby activating this important pathway. Analysis of the conditioned media by radioimmunoassay (RIA) revealed no difference in IGF-I content after 48 h between conditioned media harvested from MAC-T cells cultured on tissue culture plastic, collagen I BIOCOAT, and collagen I gel (Table 4.5). IGF-I content was not detectable in conditioned media samples from cells cultured on any of the three substrates in which 10 ng/mL or less of IGF-I was added to the media. IGF-I was detected in wells that received additions of 50 ng/mL or 100 ng/mL IGF-I; however, the detected concentrations were 40% lower for conditioned media in which 50 ng/mL IGF-I was added and 50% lower for conditioned media in which 100 ng/mL IGF-I was added; thus, a similar consumption of IGF-I for all three surfaces was observed.

TABLE 4.5

Radioimmunoassay to Detect IGF-I Concentration (ng/mL) in Cell Conditioned Media from MAC-T Cells Adhered on the Three Different Surface Substrates (Values are Mean ± SD)

IGF-I Added	Plastic	Collagen I BIOCOAT	Collagen I Gel
100	56 ± 6	72 ± 21	63 ± 15
50	32 ± 1	30 ± 4	30 ± 7
10	N/D	N/D	N/D
0	N/D	N/D	N/D

IGF-I concentration measured by radioimmunoassay (RIA) technique: concentrations of IGF-I in conditioned media for which 10 ng/mL or less of IGF-I was added was not detectable (N/D). Data shown is from two separate experiments; error bars represent standard deviations of the means.

Immunoprecipitation of the IGF-IR with subsequent Western blot analysis was performed to determine if phosphorylation of the IGF-IR was found in cells cultured on collagen I gel in the absence of IGF-I and if IGF-IR was phosphorylated in response to IGF-I. Because of the volume of the collagen I gel in plates with this surface treatment, the volume removed from collagen I gel plates after the addition of 1 mL of lysis buffer was approximately 7 mLs. Cell lysates harvested from the tissue culture plastic surface and from the collagen I BIOCOAT surface had volumes of 1 mL per plate. Only 1mL of material from each surface was processed for the Western blot. Despite the 1:7 dilution factor, lysates from collagen I gel plates yielded bands on the Western blot of detectable

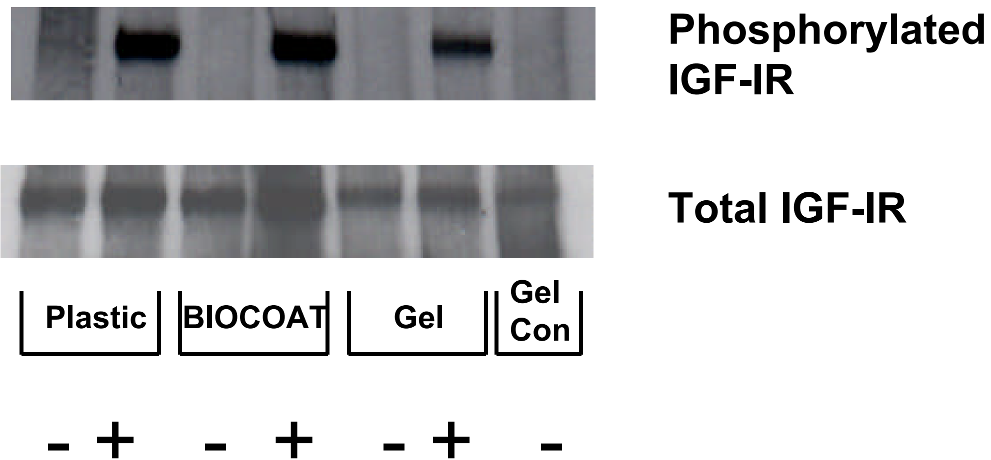


Figure 4.3. Representative Western blot of IGF-IR stimulation is shown. Blot shows darkened bands of tyrosine phosphorylation with IGF-I stimulation and bands of total IGF-IR for each substrate. Note: Samples for the plastic surface and the collagen I BIOCOAT surface are seven times more concentrated than the samples for the collagen gel substrate. Control lane is of cell-free collagen I gel processed identically to the collagen I gel with cells. Three separate experiments yielded similar results.

intensity when compared with the bands for lysates collected from tissue culture plastic and from collagen I BIOCOAT (Figure 4.3). This indicates either (1) that there are significantly more IGF-IR on the collagen I gel cells or (2) that the primary antibody used for the immunoprecipitation was limiting and that there are equivalent receptors per substrate surface. Regardless, no IGF-IR phosphorylation on the IGF-IRβ subunits was found in the absence of IGF-I on any of the surfaces. A cell-free control sample of the collagen I gel did not yield phosphorylated IGF-IR bands on the Western blot, but an unexplained band was visible for the cell-free gel control at the site of the IGF-IR band.

On visual examination of the band intensities, all three lanes with the collagen I gel surface have less intense IGF-IR bands than the two lanes for either the tissue culture

plastic surface or the two lanes for the collagen I BIOCOAT surface. The lane for the collagen I gel control band is in general darker overall than either of the lanes (without and with IGF-I) for collagen I gel with cultured cells. Comparison of the two lanes for collagen I BIOCOAT shows that the BIOCOAT lane with IGF-I is darker overall than the lane without IGF-I. The IGF-IR band in the overall darker BIOCOAT lane with IGF-I looks about twice as dark in intensity as the BIOCOAT lane without IGF-I. With this in mind (taking the dark background of the control gel lane into account), the protein band for IGF-IR in the gel control is about half the intensity of the IGF-IR bands for either of the collagen I gel lanes for which cells were cultured. It seems likely that the background IGF-IR band produced by the collagen I gel in the control lane was an alternate protein derived from the rat-tail collagen I gel substrate or even possibly that the collagen I gel samples did not fully clear of the IGF-IRβ antibody and Protein-A agarose during washes and pulse centrifugations.

The fainter bands for the collagen I gel surface compared to the other two surfaces suggests fewer IGF-IR; but since only one seventh of the total gel was analyzed, it would be expected to have only one seventh the intensity of the other bands. Densitometry measurements show the bands for the tissue culture plastic and for the collagen I BIOCOAT surfaces to be comparable (not even twice the intensity) to the measurements of the bands for the collagen I gel, but are not considered to be a definitive means for quantitative comparison due to the limitations of densitometry. The bands suggest fewer IGF-IR in the gel samples, but given the dilution factor for the material harvested from the collagen I gels, the IGF-IR content of cultured cells has not been determined.

Further investigation is needed to detect the difference in the overall quantity of IGF-IR levels on the three surface substrates. Volumes were taken into account (1 mL per surface was processed at a time), but the volumes were not based on DNA content or protein content, which would have reduced sources of variability in the comparison of the lane intensities. Based on the dilution difference between lysates from the collagen I gel and lysates from the tissue culture plastic plates and from the collagen I BIOCOAT plates and based on the phosphorylated IGF-IR bands, cells cultured on the collagen I gels appear to express higher levels of IGF-IR phosphorylation. Further, despite higher IGF-IR phosphorylation levels with IGF-I addition, a lack of endogenous phosphorylation was evident on the collagen I gel, indicating that it was not likely that autocrine signaling by the IGF-IR was the cause of the increased IGFBP-3 or the decreased IGFBP-2 levels under basal conditions on collagen I gels.

Increase in IGFBP-3 mRNA and Decrease in IGFBP-2 mRNA for Confluent IGF-I Stimulated Cells

Northern blot analysis to detect IGFBP-3 and IGFBP-2 mRNA was performed to determine if mRNA expression differences corresponded with the observed difference in the presence of these binding proteins into conditioned media (Figure 4.4 and Figure 4.5). Agreement with ligand blot analyses was not found for all cases. Not surprisingly, there was a consistent tendency for increased IGFBP-3 mRNA levels for cells with IGF-I incubation (100 ng/mL for 24 h) on all three surfaces. This corresponded with an observed increase in IGFBP-3 protein secretion as detected by ligand blot for all three surfaces (Figure 4.2). Expression of IGFBP-2 was consistently lower ($p < 0.05$) in the

presence of exogenous IGF-I on all three surfaces (Table 4.2, Table 4.4, and Chapter 5, Figure 5.2). In the absence of IGF-I addition, IGFBP-3 mRNA levels were not greater for cells on the collagen I gels despite yielding higher levels of the corresponding protein in conditioned media (Table 4.1 and Table 4.3). This was not a function of gel processing as the GAPDH levels were comparable. Cells on collagen I gel in the absence of IGF-I did show a decrease in IGFBP-2 mRNA levels which agreed with the reduced protein levels detected in the media.

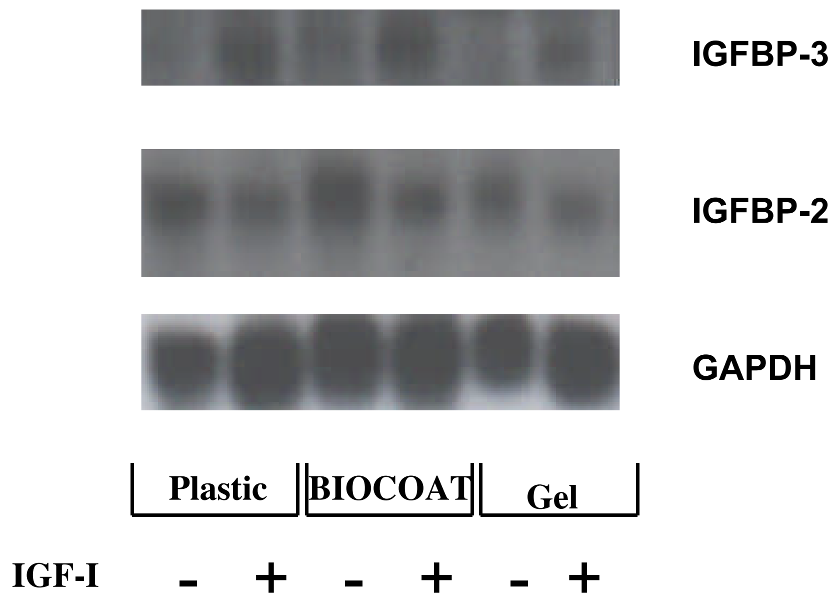
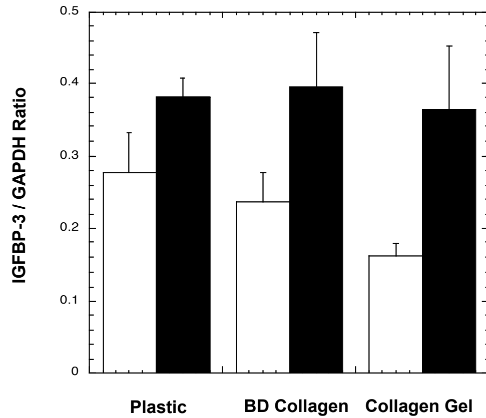


Figure 4.4. Representative Northern blot for four experiments to detect IGFBP-2 mRNA and IGFBP-3 mRNA. The GAPDH housekeeping gene is also shown. Wells with tissue culture plastic, collagen I BIOCOAT, or collagen I gel were seeded with 200,000 cells/well and incubated with or without 100 ng/mL IGF-I for 24 h.

A



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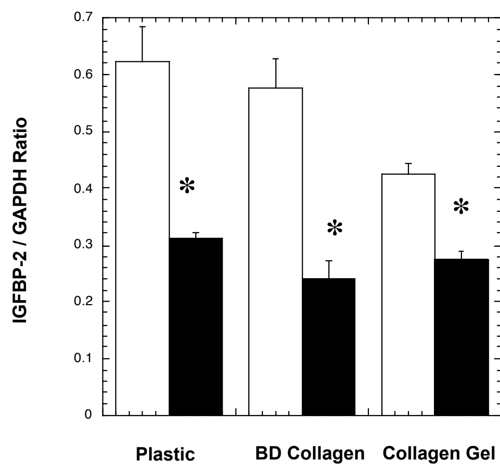

Figure 4.5. Analysis of **(A)** IGFBP-3 mRNA and **(B)** IGFBP-2 mRNA from Northern blots as measured by densitometry. Ratio of the intensity of binding protein to GAPDH housekeeping gene is shown for 24 h incubation without IGF-I (open columns) and with 100 ng/mL IGF-I (solid columns) on tissue culture plastic, collagen I BIOCOAT, or collagen I gel. Plot shows means ± SEM from three samples (two separate experiments). * Denotes significant difference from control ($p < 0.05$).

4.4 Discussion

ECM proteins have been found to impact cell proliferation and other growth factor associated cell responses for many cell types. For example, IGF-I stimulated human breast cancer cells on collagen I or on fibronectin coated wells were shown to have increased thymidine incorporation (normalized to DNA content) compared to IGF-I stimulated cells on wells coated with laminin *(17)*. The enhancement of IGF-I stimulated cell division by estrogen was reduced in MCF-7 and T47D human breast cancer cells on laminin coated wells *(17)*. Additionally, attachment of mouse mammary epithelial cells was found to be increased on collagen I and on fibronectin coated wells as compared to collagen IV and laminin coated wells *(5)*. The basal thymidine incorporation was the same for all four of these surfaces; however, thymidine incorporation with the addition of estrogen was increased only for cells on fibronectin or collagen IV coated wells. With this evidence, it was hypothesized for this study that collagen I might have an impact on the IGF-I axis of bovine mammary epithelial cells. We chose to use the MAC-T cell line for our work.

First, the secretion of IGFBP-3 and IGFBP-2 by MAC-T cells on collagen I was examined. IGFBP-3 is known to inhibit IGF-I activity in these cells *(11)*, and quantification of the difference in secretion for cells on collagen I compared to tissue culture plastic was sought. In a similar study with embryonic avian skeletal muscle cells, cells were either embedded in a collagen I gel or adhered on a collagen I coated plastic well *(15)*. Perrone, *et al.* (1995) found that the embedded cells produced 2 to 5 times the amount of IGFBP-2 and autocrine IGF-I as cells adhered to the collagen I coat. In

another study, collagen I coated wells were found to increase secretion of IGFBP-3 and IGFBP-2 by mouse mammary epithelial cells with the addition of IGF-I (300ng/ml) compared to fibronectin coated wells *(18).* In our present work, we found that MAC-T cells adhered on a collagen I gel substrate showed increased secretion of IGFBP-3 and decreased secretion of IGFBP-2 into conditioned media as compared to MAC-T cells adhered either on tissue culture plastic wells or on collagen I pre-coated wells. IGFBP-3 secretion was further increased when cells were stimulated with IGF-I, which was anticipated from previous studies *(13)*, but IGFBP-3 levels were increased even more so with collagen I gel.

Baseline IGFBP-2 secretion was clearly greater than IGFBP-3 secretion for all conditions in this study, as was expected for these cells and as has previously been shown *(13)*. Ligand blot analysis showed significantly less IGFBP-2 for cells on the collagen I gel; however, no measurement was made to determine if IGFBP-2 or IGFBP-3 was adsorbed/absorbed in the gel and therefore not included in the conditioned media used for ligand blots. Cell-free preparations of the collagen I gel were exposed to media with serum for the same time period, and the "conditioned" media was analyzed by ligand blot for a background signal. Faint bands for IGFBP-2 and for IGFBP-3 were detected on the ligand blots for the cell-free collagen I gel. It is possible that some secreted binding proteins or binding proteins from growth media might be retained in the gel in the cell systems, resulting in fainter bands than would have occurred without binding. Moreover, IGFBP-3 is known to bind to collagen *(19)*, and bound IGFBPs from original growth media that leach into the conditioned media might account for the background signals

from rat-tail collagen I gel. IGFBP-2 on the other hand, does not bind to collagen I *(20)*. Regardless, a measurable increase in the IGFBP-3 to IGFBP-2 ratio occurred in the conditioned media from cells adhered on the gels, as can be intuited from Tables 4.1-4.4 giving us reason to believe that the collagen I gel impacts the IGF-I signaling pathway in terms of binding protein secretion. (Analysis in terms of densitometry is discussed in Chapter 5.)

Also of note is the effect binding proteins had on the adhesion of cells. In the present study, no difference was found in DNA content for cells plated on the three different surfaces after 72 h. Other investigators have seen different results. Previous studies document not only changes in cell proliferation but also changes in cell attachment for mammary epithelial and other cells adhered on different matrix proteins and in the presence of IGF binding proteins *(21-23)*. For example, with Hs578T human breast cancer epithelial cells in serum-free media, the addition of IGFBP-2 and IGFBP-3 decreased attachment of these cells to a predominantly laminin ECM gel derived from mouse Engelbreth Holm-Swarm sarcoma (*22)*. However, in contrast, IGFBP-3 increased the attachment of the cells to fibronectin coated plates, and this effect was reversed by the addition of IGF-I (50 ng/mL). IGFBP-2 was found to decrease the breast cancer cell adhesion on laminin coated plates, fibronectin, coated plates, and the predominantly laminin gel. The binding of vitronectin to $\alpha v\beta 3$ integrins increased IGF-I stimulated proliferation of MCF-7beta3 human breast cancer cells *(24)*. The co-localization of IGFBP-2 with $\alpha v\beta 3$ integrin observed using confocal microscopy was associated with decreased tumor growth in tumors created from MCF 7 human breast cancer cells.

Clearly the composition of the ECM substrate and the presence of IGFBPs can affect cell attachment, but such changes were not observed in this study.

Once it was determined that collagen I gel increased IGFBP-3 secretion and decreased IGFBP-2 secretion, changes were sought in the IGF-I signaling pathway that might account for this. Collagen I was found to increase IGF-IR expression in mouse mammary epithelial cells *(18)* and was shown to impact the signaling pathway of the insulin receptor *(25,26)*. Insulin receptor phosphorylation was found to be unchanged by collagen attachment, but IRS-1 and IRS-2 phosphorylations in the insulin pathway were shown to decrease in mouse mammary epithelial cells. Further, these cells have been shown to have an increase in EGF receptor expression and Erk phosphorylation when adhered to collagen I *(26)*. Similarly, growth of human lung cancer cells on a bovine corneal endothelial ECM increased the expression of EGF compared to the expression of EGF by the same cells on plastic *(3)*. Radioimmunoassays were run to determine if the increase in IGFBP-3 from MAC-T cells on collagen I gel could be attributed to IGF-IR stimulation resulting from less removal or degradation of IGF-I from the media of cells on a collagen I gel. As expected, there was no evidence for endogenous secretion of IGF-I by MAC-T cells, and IGF-I was detected only in samples for which a high amount of exogenous IGF-I (50 ng/mL or more) was added. Cells on collagen I gel were not found to have different levels of IGF-I in the conditioned media. The IGF-I measured after 48 h was only 50% to 60% of the original added levels. This seems reasonable in that IGF-I was likely being incorporated and degraded. The initial step in IGF-I cell signaling was investigated here; that is, stimulation of the IGF-I receptor was investigated.

Since IGF-I was not being produced by the MAC-T cells and since IGF-I levels in the conditioned media were comparable, IGF-IR phosphorylation was investigated to reveal possible increase in IGF-IR stimulation within the cells via an autocrine or intracrine pathway to account for an increase in IGFBP-3 production. With Western blotting, IGF-IR phosphorylation was detected in MAC-T cells on all three surfaces only with the addition of IGF-I. No basal phosphorylation on collagen I was evident. Although additional investigation is necessary to quantify the IGF-IR levels in cells culture on the three different surfaces (tissue culture plastic, collagen I BIOCOAT, and collagen I gel), our results (Figure 4.3) suggest an increase in IGF-IR content in the cells cultured on a collagen I gel. This is due to the detectable level of IGF-IR from these cells even with a dilution factor of sevenfold in the analyzed material. The possibility of an increased number of receptors allows for additional stimulation of the IGF-I pathway in these cells, thereby providing an explanation for an increase in IGFBP-3 secretion.

As expected, Northern blot analysis revealed an increase in IGFBP-3 mRNA in MAC-T cells cultured on all three surfaces with IGF-I addition. (Unlike for the Western analysis in which only a portion of the collagen I gel material was processed for analysis, gel volume was not an issue for the Northern blot analysis because the mRNA was extracted from all of the harvested material on all three cell culture surfaces.) Surprisingly, an increase in message in the absence of exogenous IGF-I for cells cultured on collagen I gel compared to tissue culture plastic or collagen I BIOCOAT was not evident. IGFBP-2 mRNA was decreased with the addition of IGF-1 to the MACT cells on all three surfaces and also was decreased on the collagen I gel in the absence of IGF-I. However, IGFBP-2

protein detection is reduced similarly whether or not IGF-I is added to cells on the collagen I gel, and this was not reflected by the mRNA level. It has been previously shown that an increase in protein secretion does not necessarily correspond with an increase in mRNA expression as detected by Northern blot *(27)*. Not only is mRNA expression regulated, but other downstream changes such as proteolysis can also affect IGFBP presence *(28)*. We did not investigate further steps, but this would be of future interest.

The experiments to detect IGF-IR phosphorylation, IGF-I production or IGF-I presence in media, cell DNA content, and binding protein mRNA did not reveal a mechanism for altered IGFBP-3 and IGFBP-2 secretion behavior by MAC-T cells cultured on collagen I gel. Since IGFBP-3 is secreted in response to IGF-I receptor stimulation by IGF-I, one possibility is that the increased IGFBP-3 secretion on collagen I gel might result from cross-reactivity of integrins and the IGF-I signaling pathway. Further investigation of binding protein secretion behavior by the MAC-T cells on ECM matrices and other steps in the IGF-I signaling pathway remain to be examined**.** Integrins are the primary cell surface receptors for ECM proteins and are thought to interact with several growth factor pathways including the insulin-like growth factor-I (IGF-I) pathway *(2,19,25,27,30-33)*. For example, a matrix-specific connection has been demonstrated between apoptosis via the insulin signaling pathway and integrin signaling *(25)*. Inhibition of insulin signaling has been shown for mouse mammary epithelial cells grown on collagen I gel as compared to cells grown on fibronectin or Engelbreth-Holm-Swarm matrix *(26,34)*. That the IGF-I signaling pathway of MAC-T cells might be impacted by the presence of collagen I as a

surface substrate was investigated here in order to follow with studies as to how collagen I might specifically interact with the cells to induce these changes. Since the collagen I pre-coated wells did not increase IGFBP-3 as effectively as the collagen I gel did, this might also suggest that cell shape or that spatial presentation of the collagen may be critical. Other researchers have found that cell shape, regardless of the ECM protein used, will dictate cell survival on a surface *(6)*. Although the mechanism remains unknown, it would be of interest to investigate how cell shape and collagen I might impact IGFBP secretion.

4.5 Materials and Methods

Cell Culture

MAC-T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10 mL/L of antibiotic-antimycotic (100X), 1 mg/L gentamicin, 0.04 *M* $NaHCO_3$, and 10% fetal bovine serum (FBS), all purchased from Gibco (Grand Island, NY). Enzyme Free Cell Dissociation Solution was purchased from Specialty Media (Phillipsville, NJ). Calcium-free Dulbecco's phosphate buffered saline (DPBS) and trypsin (2.5% lyophilized) were also purchased from Gibco. Cells were seeded at concentrations of 50,000 cells/well or 200,000 cells/well on 24-well plates. Three different substrates on the 24-well plates were investigated: tissue culture plastic wells, Becton Dickinson collagen I (rat-tail) BIOCOAT wells, or 300 μL collagen I gels (rat-tail, in-house preparation) *(35)*. The very thin coating of collagen I in the BIOCOAT well was not visible, but an estimate of the thickness can be calculated. The goal for an in-house preparation of a thin collagen I coating on plastic was to deposit a layer of 5 μg/ cm^2 of commercially prepared collagen I from Becton Dickinson into the plastic well of a 24-well plate with a growth surface area of 2 cm^2/well using instructions from the manufacturer. Since the triple helix of collagen I is 300 nm in length, and 1.5 nm in diameter and has an approximate molecular weight of 466,000 g/mol, this would result in an approximate thickness of 0.04 mm of collagen I . For quality assurance purposes, it was decided to purchase the commercially prepared plates from Becton Dickinson with non-visible thin layers of collagen I. The thicker collagen I gel in a 24-well plate was visible to the eye, and the calculated thickness of the gel was 1.5 mm. Cells were used

between passages of 2 and 25. No tests were performed to rule out mycoplasmal contamination in this established cell line, however cells showed consistent morphology and growth behavior during the course of this research project. Cell cultures were observed under the microscope for a characteristic “cobblestone” appearance; however, no photos or measurements were made to determine if cells infiltrated the collagen I gel surface.

DNA Assay

Cells were lysed with 0.25% trypsin and Enzyme Free Cell Dissociation Solution. A 2x PBS solution (0.05 *M* Na_2HPO_4, 2 *M* NaCl, 0.002 *M* $Na_2EDTA\text{-}2H_2O$) was added to the cell material, and the solution was sonicated to further break cells to release DNA into the solution. Hoechst 33258 Dye (Hoechst Celanese) was added to prepared samples, and the samples were measured for DNA content on a Hoefer DyNA Quant fluorometer (Pharmacia Biotech).

Ligand Blot

Cells were seeded in complete media and allowed to adhere over the next 24 h. Media were then removed and replaced with DMEM without FBS. After 24 h of serum starvation, IGF-I (Peprotech, Rocky Hill, NJ) was added in concentrations of 0, 1, 5, 10, 50, and 100 ng/well. After 48 h of media conditioning, the media samples were removed, centrifuged at 1000 g for 10 min, lyophilized, and reconstituted with de-ionized water and SDS buffer to run on 12.5% SDS-PAGE gels as previously described *(36)*. Proteins were transferred to nitrocellulose membranes (NitroPure, Osmonics, Inc., Westborough,

MA) and probed with [^{125}I]IGF-I. Each membrane was placed in a cassette with X-OMAT^{TM} AR scientific imaging film (Eastman Kodak Company, NY) and allowed to expose 3-5 days. Band intensities were determined with densitometry software (GelWorks 1D Intermediate version 4.01, NonLinear Dynamics, Ltd.). The cells were lysed with 0.25% trypsin and Enzyme Free Cell Dissociation Solution and assayed for DNA content to compare the final DNA content for cells with and without added IGF-1 on all three substrate surfaces. In addition, as a control, the effect of serum addition on the three different substrates on ligand blot results was investigated. IGFBP-3 and IGFBP-2 bands were not detectable in ligand blots from cell-free systems on plastic or on collagen I BIOCOAT (data not shown). Analysis of conditioned media from cell-free collagen I gel exposed to 10% FBS resulted in detection of faint IGFBP-3 and IGFBP-2 bands; however, these bands were of less intensity than corresponding bands from conditioned media from cells adhered on the collagen I gel.

Radioimmunoassay

Samples of conditioned media were lyophilized and reconstituted in de-ionized water. IGF-I was extracted with an extraction buffer (87.5% ethanol, 12.5% 2*N* HCl) and neutralized with 0.855 *M* Tris base. Extracted samples were stored at -20ºC until ready to assay. To assay, extracted samples were diluted with RIA buffer (30 m*M* sodium phosphate monobasic, 10 m*M* EDTA, 0.02% sodium azide, 0.1% BSA (0.1% RIA Grade, Sigma), 9 mg/L phenol red (Sigma), 0.05% Tween 20, 200 mg/L protamine sulfate (Grade 1 SO_4). Primary antibody (mouse anti-IGF-I) (generous gift from Novo Nordisk A/S, Gentofte, Denmark) and ^{125}I-IGF-I tracer was added to the samples for 24 h

incubation at 4ºC. After 24 h, secondary antibody (goat anti-mouse) (Sigma) was added to the samples. Samples were allowed to incubate 72 h. Ice cold double distilled phosphate buffered saline (0.01*M*, pH 7.4) was added to all samples, the samples were centrifuged (2000 x g) for 30 min at 4ºC, and the supernatant in the tubes was discarded. The residual radioactivity in each tube was counted on a gamma counter and was compared with a standard curve for IGF-I content.

Western Blot

Cells were seeded at 6 million cells per plate for confluent adherence on 100 mm tissue culture plastic petri dishes and allowed to adhere over the next 24 h. After 48 h of serum starvation, cells were stimulated with 100 ng/mL IGF-I for 14 min. Cells were then lysed with a lysis buffer (20 m*M* HEPES, 10 m*M* sodium fluoride, 1 m*M* sodium vanadate, 500 m*M* sodium chloride, 5 m*M* EDTA, 1% Triton X-100, 10% glycerol, 1% protease inhibitor cocktail (Sigma Aldrich)) and removed from plates using a cell scraper. Petri dishes with the collagen I gel yielded seven times the volume of lysate material due to the gel than did the petri dishes with tissue culture plastic or BIOCOAT collagen I surfaces. Since equal volumes of samples were used for the analysis, the lysates from the tissue culture plastic and the collagen I BIOCOAT plates were seven times more concentrated than lysate from the collagen I gel plates. Lysates were centrifuged at 14,000 g for 15 min to remove particulates and then frozen at -70ºC until ready to process. Lysate was immunoprecipitated with primary antibody (IGF-IRβ (C20) from Santa Cruz) for 1 h at 4˚C on a platform rocker. Protein A-agarose suspension was then added for an additional 1 h incubation. The lysate/protein A-agarose suspensions were pulse centrifuged for 5

sec at the 14,000g setting to pellet the beads. The pellets were washed twice with lysis buffer and centrifuged after each wash. Supernatant was discarded and pellets were resuspended in 1x Laemmli buffer. Tubes were placed in a 100 C heat block for 2 min to denature the proteins. Samples were run on a 10% Tris-Glycine precast gel. After SDS-PAGE electrophoresis, the proteins were transferred to a PVDF membrane. Membranes were probed with P-TYR-100 antibody (NEB Products) to detect phosphorylation in the IGF-IR bands. Membranes were developed with SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Membranes were placed in cassettes to expose Kodak BioMax™ film (Eastman Kodak Co., Rochester, NY). Membranes were then stripped and re-probed for IGF-IR (IGF-IRβ (C20), Santa Cruz). Band intensities were determined with densitometry software (GelWorks 1D Intermediate version 4.01, NonLinear Dynamics, Ltd.).

Northern Blot

Cells were seeded in complete media and allowed to adhere over the next 24 h. Media were then removed and replaced with DMEM without FBS. After 48 h of serum starvation, IGF-I was added at concentrations of 0, 1, 5, 10, 50, and 100 ng/well. After an additional 24 h of incubation at 37ºC and 5% CO_2, the cells were harvested with TRI REAGENT® (Molecular Research Center, Inc., Cincinnati, OH). The harvested cells were stored at -70ºC until RNA isolation was performed. The RNA isolation procedure as recommended by the manufacturer of TRIzol reagent (Gibco Life Technologies) was followed.

Briefly, RNA samples were electrophoresed on a 1% agarose gel and then were transferred to a Hybond N^+ membrane (Amersham Pharmacia Biotech). The membrane was cross-linked in a UV crosslinker and stored at -20ºC until ready to probe. Probes were prepared with the Promega Prime-a-Gene kit (Promega) using [^{32}P]dATP and probe inserts for IGFBP-2, IGFBP-3, and the GAPDH housekeeping gene. The membranes were probed, stripped, and re-probed to detect IGFBP-3 and IGFBP-2 levels for each sample. Band intensities were determined with densitometry software (GelWorks 1D Intermediate version 4.01, NonLinear Dynamics, Ltd.).

Statistical Analysis

For ligand blot and Northern blot densitometry results, an ANOVA analysis was completed using the GLM procedure of SAS (SAS Institute, 1989). The Bonferroni (Dunn) *T* test was performed to determine significance of differences between treatments.

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4.7 References

1. Howlett, A., Bailey, N., Damsky, C., Petersen, O., and Bissel, M. (1995). Cellular growth and survival are mediated by beta 1 integrins in normal human breast epithelium but not in breast carcinoma. J. Cell Sci. **108**, 1945-1957.
1. Moghal, N. and Neel, B.G. (1998). Integration of growth factor, extracellular matrix, and retinoid signals during bronchial epithelial cell differentiation. *Mol. Cell Biol.* **18**, 6666-6678.
1. Pavelic, K., Antonic, M., Pavelic, L., Pavelic, J., Pavelic, Z., and Spaventi, S. (1992). Human lung cancers growing on extracellular-matrix–expression of oncogenes and growth-factors. *Anticancer Res.* **12**, 2191-2196.
1. Wicha, M.S., Lowrie, G., Kohn, E. et al. (1982). Extracellular matrix promotes mammary epithelial growth and differentiation in vitro. *Proc. Natl. Acad. Sci. USA.* **79**, 3213-3217.
1. Xie, J. and Haslam, S.Z. (1997). Extracellular matrix regulates ovarian hormone-dependent proliferation of mouse mammary epithelial cells. *Endocrinol.* **138,** 2466-2473.
1. Berry, S.D.K., Nielsen, M.S.W., Sejrsen K., Pearson, R.E., Boyle, P.L, and Akers, R.M. (2003). Use of an immortalized bovine mammary epithelial cell line (MAC-T) to measure the mitogenic activity of extracts from heifer mammary tissue: effects of nutrition and ovariectomy. Domest. Anim. Endocrinol. **25**, 245-253.
1. Kimmins, S. and MacLaren, L.A. (1999). Cyclic modulation of integrin expression in bovine endometrium. *Biol. Reprod*. **61**, 1267-1274.

1. Huynh, H.T., Robitaille, G., and Turner, J.D. (1991). Establishment of bovine mammary epithelial cells (MAC-T): an in vitro model for bovine lactation. *Exp. Cell Res.* **197**, 191-199.

1. Politis, I., Zavizion, B., White, J.H., Goldberg, J.J., Baldi, I., and Akers, R.M. (1995). Hormonal and extracellular-matrix regulation of plasminogen-activator in a bovine mammary epithelial-cell line. *Endocrine* **3**, 345-350.

1. Robinson, R.M., Akers, R.M., and Forsten, K.E. (2000). Real-time detection of IGF-I stimulation of the MAC-T bovine mammary epithelial cell line. *Endocrine* **13**, 345-352.

1. Blakesley, V.A., Scrimgeour, A., Esposito, D., and LeRoith, D. (1996). Signaling via the insulin-like growth factor-I receptor: does it differ from insulin receptor signaling? Cytokine Growth Factor Rev. **7**, 153-159.

1. Romagnolo, D., Akers, R.M., Byatt, J.C., Wong, E.A., and Turner, J.D. (1994). Regulation of expression of IGF-I–induced IGFBP-3 and IGF-I receptor by constitutive vs regulated expression of recombinant IGF-I in transfected mammary epithelial cells. *Endocrine J.* **2**, 375-384.

1. Jones, J.I. and Clemmons, D.R. (1995). Insulin-like growth factors and their binding proteins: biological actions. *Endocr. Rev*. **16**, 3-34.

1. Chen, C.S., Mrksich, M., Huang, S., Whitesides, G.H., and Ingber, D.E. (1997). Geometric control of cell life and death. *Science.* **276**, 1425-1428.

1. Perrone, C.E., Fenwick-Smith, D., and Vandenburgh, H.H. (1995). Collagen and stretch modulate autocrine secretion of insulin-like growth factor-1 and insulin-

like growth factor binding proteins from differentiated skeletal muscles. *J. Biol. Chem*. **270**, 2099-2106.

1. Grill, C.J. and Cohick, W.S. (2000). Insulin-like growth factor binding protein-3 mediates IGF-I action in a bovine mammary epithelial cell line independent of an IGF interaction. *J. Cell Physiol.* **183**, 273-283.
1. Woodward, T.L., Lu, H.L., and Haslam, S.Z. (2000). Laminin inhibits estrogen action in human breast cancer cells. *Endocrinol.* **141**, 2814-2821.
1. Woodward, T.L., Xie, J.W., Fendrick, J.L., and Haslam, S.Z. (2000). Proliferation of mouse mammary epithelial cells in vitro: interactions among epidermal growth factor, insulin-like growth factor I, ovarian hormones, and extracellular matrix proteins. *Endocrinol.* **141**, 3578-3586.
1. Liu, B., Weinzimer, S.A., Gibson, T.B., Mascarenhas, D., and Cohen, P. (2003). Type I alpha collagen is an IGFBP-3 binding protein. *Growth Horm. IGF Res.* **13**, 89-97.
1. Conover, C.A. and Khosia, S. (2003). Role of extracellular matrix in insulin-like growth factor (IGF) binding protein-2 regulation of IGF-II action in normal human osteoblasts. *Growth Horm. IGF Res.* **13**, 328-335.
1. Mauro, L., Sisci, D., Bartucci, M., Salerno, M., Kim, J., Tam, T., Guvakova, M.A., Ando, S., and Surmacz, R.J. (1999). SHC-a5b1 integrin interactions regulate breast cancer cells adhesion and motility. *Exp. Cell Res.* **252**, 439-448.
1. McCaig, C., Perks, C.M., and Holly, J.M.P. (2002). Intrinsic actions of IGFBP-3 and IGFBP-5 on Hs578T breast cancer epithelial cells: inhibition or accentuation

of attachment and survival is dependent upon the presence of fibronectin. *Journal of Cell Sci.* **115**, 4293-4303.

1. Nakamura, Y., Yamamoto, M., Sakamoto, K., Ohta, K., Umeda, A., Tsukamoto, T., and Nakashima, T. (2001). Growth factors, extracellular matrix components and cell adhesion molecules in Warthin's tumor. *J. Oral Pathol. Med.* **30**, 290-295.
1. Pereira, J.J., Meyer, T., Docherty, S.E., Reid, H.H., Marshall, J., Thompson, E.W., Rossjohn, J., and Price, J.T. (2004). Bimolecular interaction of insulin-like growth factor (IGF) binding protein-2 with alpha v beta 3 negatively modulates IGF-I mediated migration and tumor growth. *Cancer Res.* **64**, 977-984.
1. Farrelly, N., Lee, Y.J., Oliver, J., Dive, C., and Streuli, C.H. (1999). Extracellular matrix regulates apoptosis in mammary epithelium through a control on insulin signaling. *J. Cell Biol.* **144**, 1337-1347.
1. Perks, C.M., Gill, Z.P., Newcomb, P.V., Holly, J.M.P. (1999). Activation of integrin and ceramide signalling pathways can inhibit the mitogenic effect of insulin-like growth factor I (IGF-I) in human breast cancer cells. *Brit. J. Cancer* **79**, 701-706.
1. Voge, J.L., Santiago, C.A.T., Aad, P.Y., Goad, D.W., Malayer, J.R., and Spicer, L.J. (2004). Quantification of insulin-like growth factor binding protein mRNA using real-time PCR in bovine granulosa and theca cells: effect of estradiol, insulin, and gonadoproteins. *Domest. Anim. Endocrinol.* **26**, 241-258.
1. Sadowski T, Dietrich, S., Koschinsky, F., and Sedlacek, R. Matrix metalloproteinase 19 regulates insulin-like growth factor-mediated proliferation,

migration, and adhesion in human keratinocytes through proteolysis of insulin-like growth factor binding protein-3. *Mol. Biol. Cell* **14,** 4569-458.

1. Butler, A.A., Yakar, S., Gewolb, I.H., Karas, M., Okubo, Y., and LeRoith, D. (1998). Insulin-like growth factor-I receptor signal transduction; at the interface between physiology and cell biology. Comparative Biochem. Physiol. **121**,19-26.
1. Kricker, J.A., Towne, C.L., Firth, S.M., Herington, A.C., and Upton, Z. (2003). Structural and functional evidence for the interaction of insulin-like growth factors (IGFs) and IGF binding proteins with vitronectin. *Endocrinol.* **144**, 2807-2815.
1. Maniero, F., Murgia, C., Wary, K.K., Curatola, A.M., Pepe, A., Blumemberg, M., Westwick, J.K., Der, C.J., and Giancotti, F.G. (1997). The coupling of alpha 6 beta 4 integrin to Ras-MAP kinase pathways mediated by Shc controls keratinocyte proliferation. *EMBO J* **16**, 2365-2375.
1. Martin, J.A. and Buckwalter JA. (2000). The role of chondrocyte-matrix interactions in maintaining and repairing articular cartilage. *Biorheology* **37**, 129-140.
1. Martin-Bermudo, M. (2000). Integrins modulate the EGFr signaling pathway to regulate tendon cell differentiation in the Drosophila embryo. *Development* **127**, 2607-2615.
1. Lee, Y.J. and Streuli,, C.H. (1999). Extracellular matrix selectively modulates the response of mammary epithelial cells to different soluble signaling ligands. *J. Biol. Chem.* **274**, 22401-22408.

1. Richards, J., Larson, L., Yang, J., Guzman, R., Tomooka, Y., Osborn, R., Imagawa, W., and Nandi, S. (1983). Method for culturing mammary epithelial cells in a rat tail collagen gel matrix. *J. Tissue Cult. Methods* **8**, 31-36.

1. Hossenlopp, P., Seurin, C., Segovia-Quinson, B., Hardouin, S., and Binoux, M. (1986). Analysis of serum insulin-like growth factor binding proteins using western blotting: use of the method for titration of binding proteins and competitive binding studies. *Analytical Biochem.* **154**, 138-143.

CHAPTER 5

Topics related to detection of IGFBP-2 and IGFBP-3 from MAC-T cells cultured on collagen I

5.1 Part 1: Ligand blot detection of IGFBP-2 and IGFBP-3 from MAC-T cells on tissue culture plastic plates, collagen I BIOCOAT plates, and collagen I gels

5.1.1 Introduction/Methods

The ligand blot procedure (Appendix B) was used to detect IGFBP-2 and IGFBP-3 secretion by MAC-T cells cultured in 24-well plates of tissue culture plastic, in collagen I (rat-tail) BIOCOAT wells (Becton Dickinson), or on collagen I (rat-tail) 300 μL gels (in-house preparation *(1)*). A preliminary ligand blot to compare the IGFBP secretion of MAC-T cells cultured on the collagen I gel with MAC-T cells cultured on tissue culture plastic showed an increase in IGFBP-3 secretion on the collagen I gel, even in the absence of IGF-I. Preliminary ligand blots showed increased IGFBP-3 secretion on all three surfaces with the addition of IGF-I to the growth media. In addition, an increase in IGFBP-3 secretion for MAC-T cells cultured on tissue culture plastic was found to be dose-dependent on the IGF-I concentration. These results led to the design of an experiment to quantify the effect of the three different surface substrates on cell-secreted IGFBP with IGF-I added in concentrations of 0, 1, 5, 10, 50, and 100 ng/mL. Two cell seeding densities were investigated: 50,000 cells/well for a sub-confluent monolayer that would have actively proliferating cells and 200,000 cells/well for a confluent monolayer that would no longer have actively proliferating cells due to contact inhibition *(2-4)*. This experimental design was intended to allow the examination of two separate conditions in

the cell life stage to note possible differences in IGFBP secretion pattern under the two different conditions. Because only fifteen lanes per gel were run for a single ligand blot, several gels were run for the IGFBP secretion comparisons. Unfortunately, use of this ligand blot protocol with subsequent quantification using densitometry software did not provide the precision necessary to quantify IGF-I dose-dependent IGFBP-2 and IGFBP-3 secretion on the different surfaces. In this section, the procedure and the analysis will be outlined.

It was theorized that IGFBP secretion by MAC-T cells on the three different surface substrates could be compared by (1) seeding cells at a given cell seeding density on the different substrates, (2) collecting conditioned media containing the cell-secreted IGFBP, (3) running known amounts of conditioned media on gels and transferring the binding proteins to a membrane, (4) probing for the binding proteins, (5) exposing film to the radioactive membranes, and (6) measuring the intensity of the resultant bands using densitometry software (full details in Appendix B). Cell number was taken into account for each resultant band by measuring the DNA content of the cells from which the conditioned media was taken. Intensity of the resultant bands was calculated as the ratio of band intensity to DNA concentration. In practice, experimental error inherent in the combined measurements was too great to pool all of the data from the different blots to achieve a quantitative comparison of IGFBP secretion from MAC-T cells cultured on the three different surface substrates. Comparisons of IGFBP bands on individual blots showed consistent patterns from blot to blot. For example, IGFBP-3 secretion was

increased and IGFBP-2 secretion was decreased in MAC-T cells on a collagen I gel with the addition of IGF-I compared to MAC-T cells on tissue culture plastic.

Cell-free control wells containing collagen I gel were treated under the same conditions as the wells containing cultured cells. The IGFBP bands on the ligand blot resulting from media taken from the cell-free control wells were presumably a result of residual protein from incubation of the plates with media containing fetal bovine serum (FBS) prior to serum starvation. To create a stock of this control media for all the blots in the experiment, a 24-well plate of cell-free collagen I gel (300 μL/well) was incubated with DMEM + 10% FBS (1 mL/well) for 24 h, identical to conditions used in the cell studies. After 24 h, the media was removed, and 1 mL/well of plain DMEM was added. The collagen I gel was allowed to "condition" the media over the next 48 h. The media was then pooled, frozen to -20˚C, thawed, vortexed, and aliquoted into 24 microcentrifuge tubes for lyophilization. The lyophilized samples of this one-time prepared batch for controls were stored at 4˚C to be reconstituted as needed for each experimental run. The purpose of the control was twofold: 1) the intensity of the collagen I gel control band was subtracted out of the intensity of bands from cells cultured on the collagen I gel for each blot, and 2) the intensity of the control gel band was used as a standard to compare band intensities between the different blots.

In addition to the cell-free collagen I gel controls, cell-free tissue culture plastic wells and cell-free collagen I BIOCOAT wells were incubated with growth media under the same experimental conditions and then allowed to "condition" serum-free media.

“Conditioned media” from the tissue culture plastic wells and BIOCOAT wells did not yield background signals on the ligand blots. Only “conditioned media” from the cell-free collagen I gel yielded IGFBP-2 and IGFBP-3 bands on a ligand blot. This suggested that the collagen I gel retained small amounts of IGFBP from the growth media, the intensities of which should be subtracted out from results for cells cultured on the collagen I gel, although we note that Western analysis was not performed to confirm protein identification.

Ligand blots were set up with a total of fifteen lanes per blot. The first lane was reserved for a commercial molecular weight ladder marker (Bio-Rad, USA), and the next twelve lanes were used for three separate treatments in replicates of four. The last two lanes were reserved for the cell-free collagen gel control. Each blot was run with either one or two control lanes. For example, one blot had the configuration shown in Table 5.1.1. The corresponding ligand blot is shown in Figure 5.1.1.

To compare IGFBP-2 and IGFBP-3 secretion by MAC-T cells grown on the three different substrates and with different concentrations of IGF-I, the cells were harvested for DNA content at the same time that conditioned media was collected for ligand blot analysis. IGFBP-2 and IGFBP-3 band intensity for each treatment was divided by the cell DNA content which was measured using a DNA fluorometer. To relate one ligand blot to the next via the cell-free collagen gel conditioned media control, the intensity of IGFBP-2 and IGFBP-3 bands for each lane as measured by densitometry software was divided by the control values for IGFBP-2 and IGFBP-3 bands on each blot, respectively.

Lane #	*Treatment*	*Substrate*	*Seeding density*
1	molecular weight ladder	N/A	N/A
2	10 ng/mL IGF-I	tissue culture plastic	200,000
3	10 ng/mL IGF-I	tissue culture plastic	200,000
4	10 ng/mL IGF-I	tissue culture plastic	200,000
5	10 ng/mL IGF-I	tissue culture plastic	200,000
6	50 ng/mL IGF-I	tissue culture plastic	200,000
7	50 ng/mL IGF-I	tissue culture plastic	200,000
8	50 ng/mL IGF-I	tissue culture plastic	200,000
9	50 ng/mL IGF-I	tissue culture plastic	200,000
10	100 ng/mL IGF-I	tissue culture plastic	200,000
11	100 ng/mL IGF-I	tissue culture plastic	200,000
12	100 ng/mL IGF-I	tissue culture plastic	200,000
13	100 ng/mL IGF-I	tissue culture plastic	200,000
14	cell-free control	collagen I gel	0
15	cell free control	collagen I gel	0

Table 5.1.1. Example configuration of lanes for a single ligand blot. Each blot has one lane for a commercial molecular weight ladder, four lanes for experiment replicates, and two lanes for cell-free collagen I gel control. Multiple blots were made to obtain replicates for all three surface substrates (tissue culture plastic, collagen I BIOCOAT, collagen I gel) with different concentrations of IGF-I (0, 1, 5, 10, 50, and 100 ng/mL) at cell seeding densities of 50,000 and 200,000 cells/well.

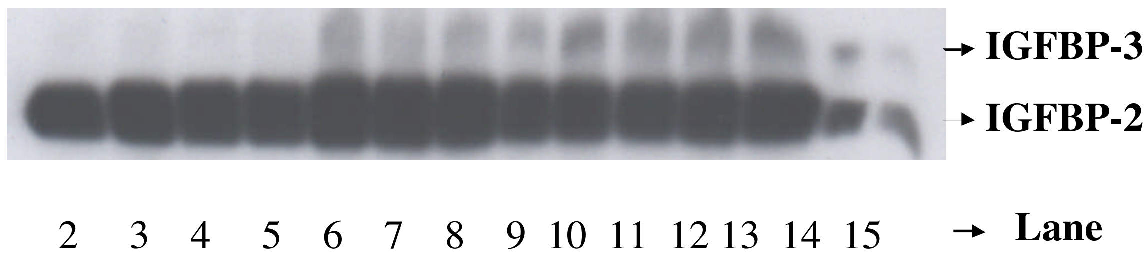

Figure 5.1.1. Ligand blot with lanes corresponding to the configuration shown in Table 5.1.1 for conditioned media from 200,000 MAC-T cells per well on tissue culture plastic. Lanes 2 through 5 were treated with 10 ng/mL IGF-I, lanes 6 through 9 were treated with 50 ng/mL IGF-I, and lanes 10 through 13 were treated with 100 ng/mL IGF-I. Lane 1 was reserved for a commercial molecular weight ladder; lanes 14 and 15 were reserved for the cell-free collagen I gel control.

5.1.2 Results

Analysis 1

In actuality, the values for IGFBP-2 and IGFBP-3 intensities did not match up very well from one ligand blot to the next as is shown by the results from two separate blots in Figure 5.1.2 and Figure 5.1.3 and the corresponding densitometry intensities shown in Table 5.1.2. Within each ligand blot, replication of four wells of the same treatment in a row showed consistency within treatments as shown in each single blot in (Figure 5.1.1, Figure 5.1.2, and Figure 5.1.3). The variation could not be accounted for by DNA data; DNA data was consistent for samples collected from the three different surfaces in all samples and increased with the addition of IGF-I (pooled data from multiple samples shown in Chapter 4, Figure 4.1).

The following tables illustrate the lack of agreement of data from two separate ligand blots for MAC-T cells seeded at 50,000 cells/well on collagen I BIOCOAT wells with increasing amounts of IGF-I addition. In Table 5.1.2 , the intensities (as measured by densitometry) of the IGFBP bands for each blot is shown. In Table 5.1.3, the data is shown on a cellular DNA per well basis. In other words, the relative intensity of each band as measured by densitometry software in Table 5.1.2 is divided by the corresponding DNA content of the cells in each well as measured by a DNA fluorometer. To further normalize data from the two blots, the values for IGFBP-2 and IGFBP-3 were divided by the intensities of the corresponding IGFBP bands of the cell-free collagen I gel control on each blot. (The cell-free collagen I gel was used as the control to tie together all of the blots, regardless of experimental surface substrate. Each blot had either one or two control lanes.) The values shown in Table 5.1.4 have been calculated as multiples of the intensities of the control bands.

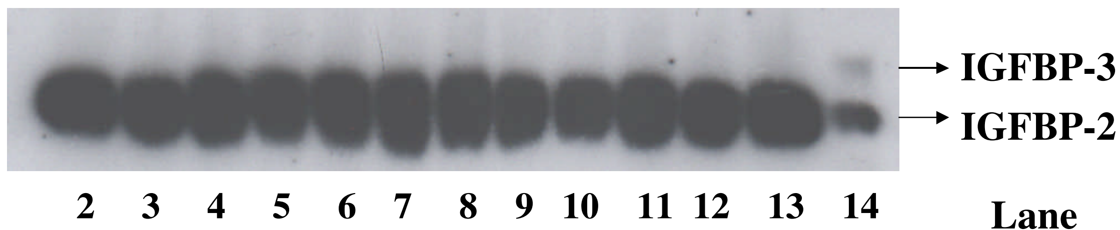

Figure 5.1.2. Ligand blot with lanes corresponding to the densitometry measurements shown in Table 5.1.2 for conditioned media from 50,000 MAC-T cells per well on collagen I BIOCOAT. Lanes 2 through 5 were treated with 0 ng/mL IGF-I, lanes 6 through 9 were treated with 1 ng/mL IGF-I, and lanes 10 through 13 were treated with 5 ng/mL IGF-I. Lane 14 shows the cell-free collagen I gel control. (Only one control lane was run in this blot.)

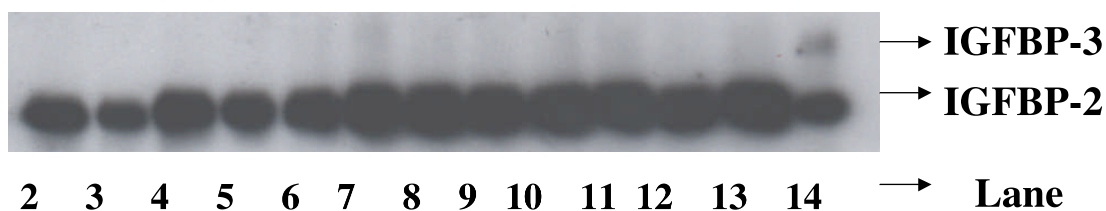

Figure 5.1.3. Ligand blot with lanes corresponding to the densitometry measurements shown in Table 5.1.2 for conditioned media from 50,000 MAC-T cells per well on collagen I BIOCOAT. Lanes 2 through 5 were treated with 10 ng/mL IGF-I, lanes 6 through 9 were treated with 50 ng/mL IGF-I, and lanes 10 through 13 were treated with 100 ng/mL IGF-I. Lane 14 shows the cell-free collagen I gel control. (Only one control lane was run in this blot.)

IGF-I (ng/mL)	*IGFBP-2 Band Average Intensity of 4 wells (± SE)*	*IGFBP-3 Band Average Intensity of 4 wells (± SE)*	*Cell-free Collagen I Gel IGFBP-2 Band Control Intensity*	*Cell-free Collagen I Gel IGFBP-3 Band Control Intensity*
0	7.30 (± 0.13) x 10^7	1.14 (± 0.07)x 10^7	2.19 x 10^7	1.04 x 10^7
1	6.84 (± 0.04)x 10^7	1.70 (± 0.10) x 10^7	2.19 x 10^7	1.04 x 10^7
5	6.80 (± 0.05) x 10^7	1.68 (± 0.08) x 10^7	2.19 x 10^7	1.04 x 10^7
10	4.06 (± 0.21) x 10^7	0.43 (± 0.04) x 10^7	3.98 x 10^7	7.74 x 10^6
50	5.85 (± 0.21) x 10^7	0.31 (± 0.03) x 10^7	3.98 x 10^7	7.74 x 10^6
100	6.15 (± 0.08) x 10^7	0.60 (± 0.05) x 10^7	3.98 x 10^7	7.74 x 10^6

Table 5.1.2. IGFBP-2 and IGFBP-3 band intensities as measured by densitometer from two separate ligand blots (shown in Figure 5.1.2 and Figure 5.1.3) for MAC-T cells cultured on collagen I BIOCOAT at 50,000 cells/well. Ligand blot in Figure 5.1.2 (labeled as blot 8 in Table 5.1.7 and Table 5.1.10): 0, 1, and 5 ng/mL IGF-I. Ligand blot in Figure 5.1.3 (labeled as blot 10 in Tables 5.1.7 and 5.1.10): 10, 50, and 100 ng/mL IGF-I. Corresponding intensities of cell-free collagen I gel controls are also shown. No error bars are shown for the control intensities, as only one cell-free collagen I control lane was run on these blots.

IGF-I (ng/mL)	*DNA* (ng/mL)	*IGFBP-2 Band Average Intensity on ng/mL cellular DNA basis*	*IGFBP-3 Band Average Intensity on ng/mL cellular DNA basis*
0	73 (± 2)	9.9×10^5	1.5×10^5
1	83 (± 5)	8.2×10^5	2.0×10^5
5	90 (± 4)	7.5×10^5	1.8×10^5
10	110 (± 6)	3.6×10^5	3.8×10^4
50	160 (± 6)	3.6×10^5	1.9×10^4
100	150 (± 20)	4.0×10^5	3.9×10^4

Table 5.1.3. Data from Table 5.1.2 calculated with IGFBP-2 and IGFBP-3 band intensities on a per ng/mL cellular DNA basis from MAC-T cells cultured on collagen I BIOCOAT at 50,000 cells/well. Mean intensity ± SEM are shown for the DNA measurements. Error is not shown for the calculated average intensity on ng/mL cellular DNA basis due to combined uncertainties of the intensity and of the DNA readings.

IGF-I (ng/mL)	***IGFBP-2 Band on ng/mL cellular DNA basis normalized to cell-free collagen I gel control***	***IGFBP-3 Band on ng/mL cellular DNA basis normalized to cell-free collagen I gel control***
0	0.030	0.012
1	0.025	0.016
5	0.023	0.014
10	0.012	0.0028
50	0.012	0.0014
100	0.013	0.0029

Table 5.1.4. Ligand blot 1: Cell-free collagen I gel control normalized IGFBP-2 and IGFBP-3 band intensity on a per ng/mL cellular DNA basis from MAC-T cells cultured on collagen I BIOCOAT at 50,000 cells/well. Error bars are not shown, as the uncertainty of the single reading of cell-free collagen I gel is unknown and both the intensity and the DNA readings have separate uncertainties.

In looking at Tables 5.1.2, 5.1.3, and 5.1.4, there is a break between the quantities for 0, 1, and 5 ng/mL IGF-I and the quantities for 10, 50, and 100 ng/mL IGF-I. On closer examination, the ratios of the intensities of the IGFBP-2 band *vs*. the IGFBP-3 band on the different blots were compared. These were found to vary from 0.1 to 0.7, even though all of the samples originated from the same batch of cell-free collagen I gel conditioned media. Also, it can be observed that the blot in Figure 5.1.2 looks a little more exposed than the blot in Figure 5.1.3. The exposures did not match exactly given

the decay of ^{125}I over the course of the experiments. Films were visualized in the darkroom to detect readable bands by sight on the films before quenching film development. Comparison of the data on the two blots shows that this procedure did not give the precision needed to produce a comprehensive data array for the points as desired; a repeat of the experiment did not yield improved results.

Analysis 2

Since direct comparisons of the effect of the surface on the MAC-T cells could not be made by quantifying IGFBP-2 and IGFBP-3 secretion by the procedure outlined in Appendix B, the next approach was to quantify an IGFBP-3 to IGFBP-2 secretion ratio. The ratio approach did not require the same precision in adding a given volume of the protein sample to the gel since the sample was well mixed and the protein intensity ratio, not absolute intensities, were compared from gel to gel. Variation in ratios reflected either variance in the samples or limitations of the densitometry software and not variance from film exposure since both the IGFBP-2 and IGFBP-3 for each ratio was taken from the same blot. In spite of the considerable variance in ratios, the ratios of the cell-free collagen I gel controls were relatively stable, and overall conclusions about sample sets could be made by comparison with the controls. Table 5.1.5 summarizes the IGFBP-3/IGFBP-2 ratio calculated from band intensities resulting from samples of conditioned media collected from MAC-T cells cultured on tissue culture plastic and on collagen I gel at an initial seeding density of 200,000 cells/well. The increase in IGFBP-

3/IGFBP-2 becomes apparent for cells on the collagen I gel when IGF-I addition is 50 to 100 ng/mL.

IGF-I (ng/mL)	***IGFBP-3/IGFBP-2 from cells on Plastic 200,000 cells***	***IGFBP-3/IGFBP-2 from cells on Collagen I Gel 200,000 cells***	***IGFBP-3/IGFBP-2 from cell-free Control Collagen I Gel***
0	0	0.13	0.25
	0	0.15	0.18
1	0	0.51	0.25
	0	0.07	0.18
5	0	0.47	0.25
	0	0.19	0.18
10	0	0.54	0.67
	0.03	0.02	0.15
		0.39	0.80
50	0.26	0.63	0.17
	0.03	0.13	0.15
		1.04	0.21
100	0.45	0.11	0.17
	0.14	0.47	0.15
		1.09	0.21

Table 5.1.5. IGFBP-3/IGFBP-2 ratio in MAC-T conditioned media from 200,000 cells/well cultured on tissue culture plastic or on collagen I gel. Intensity of collagen I gel background control bands have been subtracted out of the IGFBP-2 and IGFBP-3 bands for conditioned media collected from cells cultured on collagen I gels prior to calculation of the IGFBP-3/IGFBP-2 ratios. Data is calculated from three independent experiments for which the data is summarized in Tables 5.1.6 through 5.1.17.

Summary of ligand blot densitometry data

Tables 5.1.6 through 5.1.17 summarize the data collected from the multiple ligand blots. Data is shown for each surface from a total of three independent experiments (each column is a separate experiment). Some data points are only available from two experiments of the three experiments, and only two columns are shown in those cases. Individual blots from all three experiments were assigned numbers from 1 to 26, with a total of twelve data points (four replicates each for three IGF-I concentrations) per blot. The average intensity calculated from four replicates with the calculated standard error of the mean are shown for each surface at each IGF-I concentration. Multiple ligand blots were required to process the samples from the three experiments. The resulting data has been tabulated into sets according to binding protein and cell seeding concentration. Assigned blot number is identified in each grouping of the data.

SET A: (Tables 5.1.6 through 5.1.8) IGFBP-2 band intensities from conditioned media of MAC-T cells initially seeded at density of 50,000 cells/well.

IGF-I (ng/mL)	*Plastic Blot 1 (Set 1)*	*Plastic Blot 2 (Set 2)*	*Plastic Blot 3 (Set 3)*
0	2.84 (± 0.03) x 10^8	1.08 (± 0.06) x 10^8	4.01 (± 0.32) x 10^7
1	2.60 (± 0.03) x 10^8	8.97 (± 0.03) x 10^7	4.20 (± 0.26) x 10^7
5	2.74 (± 0.04) x 10^8	1.06 (± 0.05) x 10^8	5.30 (± 0.29) x 10^7
Control Gel (Cell-free)	1.25 x 10^8	-	1.08 x 10^7
	Plastic Blot 4(Set 1)	*Plastic Blot 5(Set 2)*	*Plastic Blot 6 (Set 3)*
10	2.69 (± 0.07) x 10^8	1.11 (± 0.06) x 10^8	1.79 (± 0.14) x 10^7
50	2.35 (± 0.03) x 10^8	9.18 (± 0.51) x 10^7	1.54 (± 0.15) x 10^7
100	2.47 (± 0.02) x 10^8	7.81 (± 0.18) x 10^7	1.48 (± 0.31) x 10^7
Control Gel (Cell-free)	1.07 x 10^8	9.35 x 10^7	1.32 x 10^7

Table 5.1.6. Set A: IGFBP-2 band intensities on ligand blots from conditioned media of MAC-T cells initially seeded at density of 50,000 cells/well on tissue culture plastic. Mean intensity ± SEM is shown.

IGF-I (ng/mL)	*Collagen I BIOCOAT Blot 7(Set 2)*	*Collagen I BIOCOAT Blot 8 (Set 3)*
0	2.10 (± 0.12) x 10^8	7.30 (± 0.13) x 10^7
1	2.20 (± 0.06) x 10^8	6.84 (± 0.04) x 10^7
5	2.17 (± 0.02) x 10^8	6.80 (± 0.05) x 10^7
Control Gel (Cell-free)	9.62 x 10^7	2.19 x 10^7
	8.26 x 10^7	
	Collagen I BIOCOAT Blot 9 (Set 2)	*Collagen I BIOCOAT Blot 10 (Set 3)*
10	1.64 (± 0.05) x 10^8	4.06 (± 0.21) x 10^7
50	1.52 (± 0.04) x 10^8	5.85 (± 0.21) x 10^7
100	1.84 (± 0.03) x 10^8	6.15 (± 0.08) x 10^7
Control Gel (Cell-free)	6.63 x 10^7	3.98 x 10^7
	6.38 x 10^7	

Table 5.1.7. Set A: IGFBP-2 band intensities on ligand blots from conditioned media of MAC-T cells initially seeded at density of 50,000 cells/well on collagen I BIOCOAT. Mean intensity ± SEM is shown.

IGF-I (ng/mL)	*Collagen I gel Blot 11(Set 2)*	*Collagen I gel Blot 12 (Set 3)*
0	7.14 (± 0.39) x 10^7	2.42 (± 0.25) x 10^7
1	4.25 (± 0.94) x 10^7	2.62 (± 0.18) x 10^7
5	3.11 (± 0.20) x 10^7	2.02 (± 0.34) x 10^7
Control Gel (Cell-free)	4.00 x 10^7	1.98 x 10^7
	1.16 x 10^7	
	Collagen I gel Blot 13 (Set 2)	***Collagen I gel Blot 14 (Set 3)***
10	4.83 (± 0.38) x 10^7	5.71 (± 0.17) x 10^7
50	6.13 (± 0.09) x 10^7	6.84 (± 0.08) x 10^7
100	8.89 (± 0.15) x 10^7	5.67 (± 0.04) x 10^7
Control Gel (Cell-free)	6.15 x 10^7	4.08 x 10^7
	6.01 x 10^7	

Table 5.1.8. Set A: IGFBP-2 band intensities on ligand blots from conditioned media of MAC-T cells initially seeded at density of 50,000 cells/well on collagen I gel. Mean intensity ± SEM is shown.

SET B: (Tables 5.1.9 through 5.1.11) IGFBP-3 band intensities from conditioned media of MAC-T cells initially seeded at density of 50,000 cells/well.

IGF-I (ng/mL)	***Plastic Blot 1 (Set 1)***	***Plastic Blot 2 (Set 2)***	***Plastic Blot 3 (Set 3)***
0	0	0	0
1	0	0	0
5	0	0	0
Control Gel (Cell-free)	2.99×10^7	-	3.15×10^6
	Plastic Blot 4 (Set 1)	***Plastic Blot 5 (Set 2)***	***Plastic Blot 6 (Set 3)***
10	$1.09 (\pm 0.45) \times 10^7$	0	0
50	$1.66 (\pm 0.12) \times 10^7$	$1.10 (\pm 0.47) \times 10^7$	0
100	$1.41 (\pm 0.14) \times 10^7$	$2.16 (\pm 0.72) \times 10^7$	0
Control Gel (Cell-free)	2.78×10^7	5.70×10^7	1.35×10^6

Table 5.1.9. Set B: IGFBP-3 band intensities on ligand blots from conditioned media of MAC-T cells initially seeded at density of 50,000 cells/well on tissue culture plastic. Mean intensity ± SEM is shown.

IGF-I (ng/mL)	*Collagen I BIOCOAT Blot 7 (Set 2)*	*Collagen I BIOCOAT Blot 8 (Set 3)*
0	0	1.14 (± 0.07) x 10^7
1	0	1.70 (± 0.10) x 10^7
5	0	1.68 (± 0.08) x 10^7
Control Gel (Cell-free)	3.92 x 10^7	1.04 x 10^7
	3.54 x 10^7	
	Collagen I BIOCOAT Blot 9 (Set 2)	*Collagen I BIOCOAT Blot 10 (Set 3)*
10	0	4.27 (± 0.45) x 10^6
50	0	3.09 (± 0.34) x 10^6
100	0	6.00 (± 0.49) x 10^6
Control Gel (Cell-free)	2.88 x 10^7	7.74 x 10^6
	3.51 x 10^7	

Table 5.1.10. Set B: IGFBP-3 band intensities on ligand blots from conditioned media of MAC-T cells initially seeded at density of 50,000 cells/well on collagen I BIOCOAT. Mean intensity ± SEM is shown.

IGF-I (ng/mL)	***Collagen I gel Blot 11 (Set 2)***	***Collagen I gel Blot 12 (Set 3)***
0	2.36 (± 0.25) x 10^7	8.18 (± 1.15) x 10^6
1	8.40 (± 2.28) x 10^6	8.69 (± 0.53) x 10^6
5	9.38 (± 0.85) x 10^7	7.35 (± 1.33) x 10^6
Control Gel (Cell-free)	2.47 x 10^7	4.95 x 10^6
	5.10 x 10^6	
	Collagen I gel Blot 13 (Set 2)	***Collagen I gel Blot 14 (Set 3)***
10	1.22 (± 0.25) x 10^6	3.36 (± 0.13) x 10^7
50	2.34 (± 0.22) x 10^7	4.34 (± 0.17) x 10^7
100	4.75 (± 0.20) x 10^7	4.21 (± 0.10) x 10^7
Control Gel (Cell-free)	1.76 x 10^7	1.43 x 10^7
	1.40 x 10^7	

Table 5.1.11. Set B: IGFBP-3 band intensities on ligand blots from conditioned media of MAC-T cells initially seeded at density of 50,000 cells/well on collagen I gel. Mean intensity ± SEM is shown.

SET C: (Tables 5.1.12 through 5.1.14) IGFBP-2 band intensities from conditioned media of MAC-T cells initially seeded at density of 200,000 cells/well.

IGF-I (ng/mL)	***Plastic Blot 15 (Set 2)***	***Plastic Blot 16 (Set 3)***
0	1.49 (± 0.62) x 10^8	9.74 (± 0.24) x 10^7
1	2.46 (± 0.54) x 10^8	4.67 (± 0.36) x 10^7
5	2.38 (± 0.39) x 10^8	4.23 (± 0.40) x 10^7
Control Gel (Cell-free)	3.52 x 10^7	2.15 x 10^7
	3.34 x 10^7	
	Plastic Blot 17 (Set 2)	***Plastic Blot 18 (Set 3)***
10	2.27 (± 0.05) x 10^8	1.06 (± 0.57) x 10^8
50	2.32 (± 0.04) x 10^8	8.95 (± 0.11) x 10^7
100	1.97 (± 0.01) x 10^8	8.44 (± 0.05) x 10^7
Control Gel (Cell-free)	4.16 x 10^7	3.29 x 10^7
	7.43 x 10^7	

Table 5.1.12. Set C: IGFBP-2 band intensities on ligand blots from conditioned media of MAC-T cells initially seeded at density of 200,000 cells/well on tissue culture plastic. Mean intensity ± SEM is shown.

IGF-I (ng/mL)	***Collagen I BIOCOAT Blot 19 (Set 2)***	***Collagen I BIOCOAT Blot 20 (Set 3)***
0	3.52 (± 0.07) x 10^8	5.80 (± 0.15) x 10^7
1	3.13 (± 0.08) x 10^8	5.17 (± 0.20) x 10^7
5	3.80 (± 0.02) x 10^8	5.38 (± 0.17) x 10^7
Control Gel (Cell-free)	1.53 x 10^8	3.92 x 10^7
	1.86 x 10^8	
	Collagen I BIOCOAT Blot 21 (Set 2)	***Collagen I BIOCOAT Blot 22 (Set 3)***
10	3.09 (± 0.07) x 10^8	4.22 (± 0.25) x 10^7
50	2.45 (± 0.18) x 10^8	6.02 (± 0.27) x 10^7
100	3.36 (± 0.05) x 10^8	6.35 (± 0.11) x 10^7
Control Gel (Cell-free)	9.7 x 10^7	3.61 x 10^6
	7.5 x 10^7	

Table 5.1.13. Set C: IGFBP-2 band intensities on ligand blots from conditioned media of MAC-T cells initially seeded at density of 200,000 cells/well on collagen I BIOCOAT. Mean intensity ± SEM is shown.

IGF-I (ng/mL)	*Collagen I gel Blot 23 (Set 2)*	*Collagen I gel Blot 24 (Set 3)*
0	3.15 (± 0.53) x 10^7	3.67 (± 0.10) x 10^7
1	3.44 (± 0.37) x 10^7	3.97 (± 0.06) x 10^7
5	3.33 (± 0.40) x 10^7	3.67 (± 0.16) x 10^7
Control Gel (Cell-free)	1.46 x 10^7	1.43 x 10^7
	1.42 x 10^7	
	Collagen I gel Blot 25 (Set 2)	***Collagen I gel Blot 26 (Set 3)***
10	2.59 (± 0.38) x 10^7	3.09 (± 0.44) x 10^7
50	1.96 (± 0.26) x 10^7	3.42 (± 0.15) x 10^7
100	4.05 (± 0.52) x 10^7	3.64 (± 0.13) x 10^7
Control Gel (Cell-free)	1.02 x 10^7	1.71 x 10^7
	1.57 x 10^7	

Table 5.1.14. Set C: IGFBP-2 band intensities on ligand blots from conditioned media of MAC-T cells initially seeded at density of 200,000 cells/well on collagen I gel. Mean intensity ± SEM is shown.

SET D: (Tables 5.1.15 through 5.1.17) IGFBP-3 band intensities from conditioned media of MAC-T cells initially seeded at density of 200,000 cells/well.

IGF-I (ng/mL)	*Plastic Blot 15 (Set 2)*	*Plastic Blot 16 (Set 3)*
0	0	0
1	0	0
5	0	0
Control Gel (Cell-free)	1.94×10^7	4.50×10^6
	1.86×10^7	
	Plastic Blot 17 (Set 2)	***Plastic Blot 18 (Set 3)***
10	0	$3.24 (\pm 0.68) \times 10^6$
50	$5.94 (\pm 0.23) \times 10^7$	$2.34 (\pm 0.08) \times 10^6$
100	$8.89 (\pm 0.18) \times 10^7$	$1.16 (\pm 0.27) \times 10^7$
Control Gel (Cell-free)	1.17×10^7	1.26×10^7
	2.77×10^7	

Table 5.1.15. Set D: IGFBP-3 band intensities on ligand blots from conditioned media of MAC-T cells initially seeded at density of 200,000 cells/well on tissue culture plastic. Mean intensity ± SEM is shown.

IGF-I (ng/mL)	***Collagen I BIOCOAT Blot 19 (Set 2)***	***Collagen I BIOCOAT Blot 20 (Set 3)***
0	6.62 (± 0.38) x 10^7	0
1	4.92 (± 0.38) x 10^7	0
5	5.21 (± 0.31) x 10^7	0
Control Gel (Cell-free)	4.54 x 10^7	1.20 x 10^7
	Collagen I BIOCOAT *Blot 21 (Set 2)*	***Collagen I BIOCOAT Blot 22 (Set 3)***
10	3.16 (± 0.34) x 10^7	1.29 (± 0.86) x 10^6
50	5.32 (± 0.27) x 10^7	7.22 (± 0.65) x 10^6
100	5.47 (± 0.36) x 10^7	1.28 (± 0.10) x 10^7
Control Gel (Cell-free)	4.61 x 10^7	1.40 x 10^6
	2.45 x 10^7	

Table 5.1.16. Set D: IGFBP-3 band intensities on ligand blots from conditioned media of MAC-T cells initially seeded at density of 200,000 cells/well on collagen I BIOCOAT. Mean intensity ± SEM is shown.

IGF-I (ng/mL)	***Collagen I gel Blot 23 (Set 2)***	***Collagen I gel Blot 24 (Set 3)***
0	6.51(± 1.35) x 10^6	8.03 (± 0.76) x 10^6
1	4.83 (± 1.00) x 10^6	1.61 (± 0.05) x 10^7
5	6.75 (± 1.16) x 10^6	1.69 (± 0.08) x 10^7
Control Gel (Cell-free)	3.16 x 10^6	5.88 x 10^6
	2.81 x 10^6	
	Collagen I gel Blot 25 (Set 2)	***Collagen I gel Blot 26 (Set 3)***
10	3.21 (± 0.42) x 10^6	5.51 (± 1.11) x 10^6
50	2.66 (± 0.30) x 10^6	5.89 (± 0.25) x 10^6
100	1.73 (± 0.25) x 10^7	8.33 (± 0.54) x 10^6
Control Gel (Cell-free)	2.69 x 10^6	4.93 x 10^6
	3.19 x 10^6	

Table 5.1.17. Set D: IGFBP-3 band intensities on ligand blots from conditioned media of MAC-T cells initially seeded at density of 200,000 cells/well on collagen I gel. Mean intensity ± SEM is shown.

As a result of the difficulties encountered with densitometry, an additional two experiments were conducted using only 0 and 100 ng/mL IGF-I treatments of MAC-T cells on the three different surfaces (tissue culture plastic, collagen I BIOCOAT, and collagen I gel) on the same blot to see if densitometry would yield statistically significant results for the visually observed results. Analysis of the visually observed results are shown in Chapter 4, Table 4.3 and Table 4.4. Despite the experimental issues, statistical analysis of the data set shown in Figure 5.1.4 using the GLM procedure Bonferroni (Dunn) t-test (SAS Institute, Cary, NC) did confirm that the IGFBP-2 level was significantly less ($p < 0.05$) for cells cultured on the collagen I gel at both cell seeding levels. The other observed trends from the individual experiments were not found to be statistically significant when the densitometry readings were combined. An attempt was made to scale these values by dividing the value for a each reading by the reading for a baseline value; however, this likewise did not yield a statistically significant result.

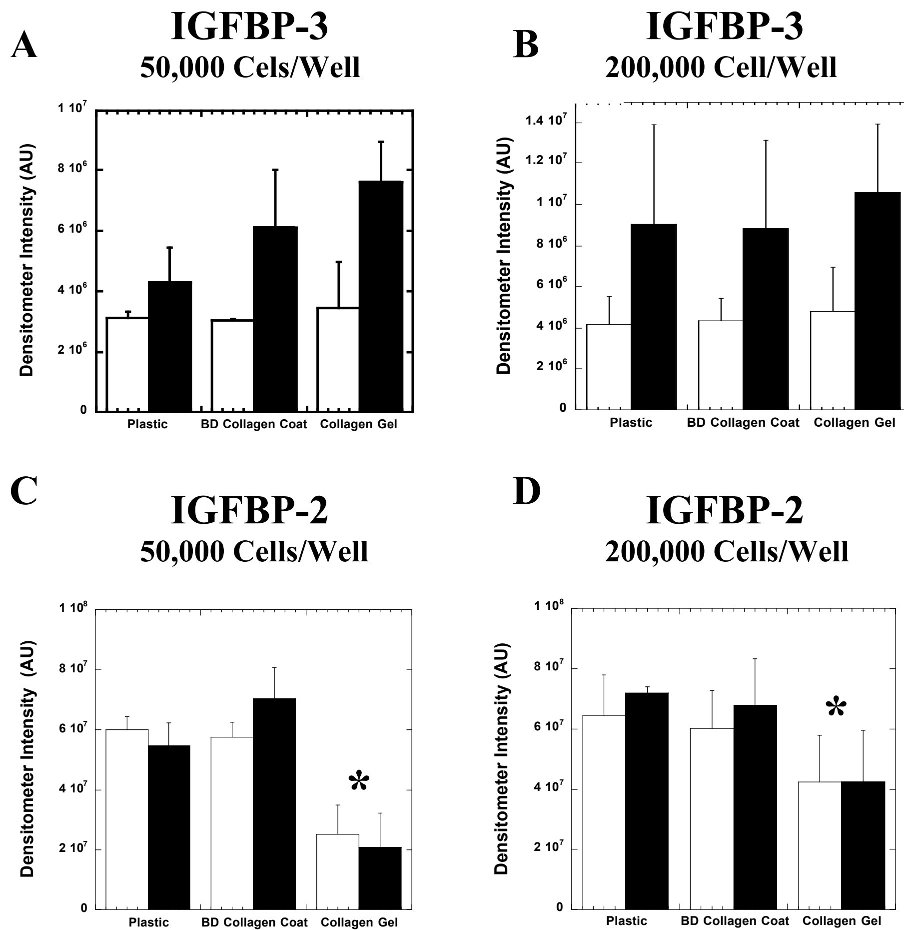

Figure 5.1.4. Average intensities of IGFBP-3 and IGFBP-2 bands on ligand blot as measured by densitometry from two experiments is summarized. Intensities from bands from cell-free gel controls were subtracted out of the collagen gel data. **(A)** IGFBP-3 from cells seeded at 50,000 cells/well. **(B)** IGFBP-3 from cells seeded at 200,000 cells/well. **(C)** IGFBP-2 from cells seeded at 50,000 cells/well. **(D)** IGFBP-2 from cells seeded at 200,000 cells/well. Cells were incubated 48 h either without IGF-I (open columns) or with 100ng/mL IGF-I (solid columns) on the tissue culture plastic, collagen I BIOCOAT, and collagen I gel. Plot shows mean intensity ± SEM from two separate experiments (two samples per experiment). * Denotes significant difference from control ($p < 0.05$).

Summary of Results

The densitometry data for the ligand blots to determine IGFBP-2 and IGFBP-3 secretion on the three surfaces (tissue culture plastic, collagen I BIOCOAT, and collagen I gel) with IGF-I (0 to 100 ng/mL) showed the following overall results:

1.) MAC-T cells cultured on tissue culture plastic wells secreted very little IGFBP-3 as compared to IGFBP-2. Blots needed a longer exposure time to quantify IGFBP-3 bands. This resulted in the over-exposure of the IGFBP-2 bands, so two separate approximate exposure times were needed to quantify and compare both IGFBP bands (Chapter 4, Figure 4.2).

2.) Addition of IGF-I to MAC-T cells cultured on tissue culture plastic showed that the increase in IGFBP-3 secretion became evident at 50 and 100 ng/mL IGF-I (Table 5.1.15, Figure 5.1.4).

3.) MAC-T cells cultured on collagen I BIOCOAT plates also secreted IGFBP-2 and IGFBP-3 (Figure 5.1.2, Figure 5.1.3, Table 5.1.2, Table 5.1.7, Table 5.1.10, Table 5.1.13, Table 5.1.16). Also, the addition of IGF-I at 50 and 100 ng/mL increased IGFBP-3 expression, as for tissue culture plastic. Results for collagen I BIOCOAT were similar to the results for tissue culture plastic as described in Chapter 4. The increase in IGFBP-3 in the absence of IGF-I was only clearly evident for cells cultured on collagen I gel (Chapter 4, Figure 4.2, Table 4.1, Table 4.2, Table 4.3, Table 4.4).

4.) MAC-T cells cultured on collagen I gels secreted IGFBP-3 in the absence of IGF-I and secreted higher amounts of IGFBP-3 and lesser amounts of IGFBP-2 in the presence of IGF-I (Figure 5.1.4 and Chapter 4, Figure 4.2, Table 4.1, Table 4.2, Table 4.3, Table 4.4).

5.1.3 Discussion

The aim of this set of experiments was to quantify the IGF-I dose-dependent IGFBP-3 secretion by MAC-T cells cultured on the three different surfaces: tissue culture plastic, collagen I BIOCOAT, and collagen I gel. Limitations of this system included the fact that a single blot yielded a comparison of only fifteen data points. Within blots, replication of four wells of the same treatment in a row showed consistency within treatments.

Several problems were identified in the attempt to quantify the IGFBP-2 and IGFBP-3 secretion by MAC-T cells on the three different surfaces. First of all, comparisons between blots were varied in part because the radioactivity of iodinated IGF-I continually decays (the half life of the ^{125}I isotope is 2 months). Each blot required a 3-day procedure after the lyophilized media was obtained, and exposure of the film to the probed blot required another 3 to 5 days. The exposure timing was imprecise, as an estimate was made as to how long to expose the film before development. If bands on the resultant developed film did not appear clear enough to the eye, a second film was exposed for a longer time period. Overly exposed, very dark bands on a film also required re-exposure of the blot with a second film, for a shorter exposure time period.

Film development was also imprecise, as it was not automated. Exposed film was placed in developer solution, and the band development was watched under a red lamp until strong bands appeared and the development process was continued by hand. The decay in radiation and imprecise development contributed to variation in band intensity from

blot to blot, but we attempted to relate bands between blots based on relative intensity levels using a control sample. It was found that the "conditioned media" from a cell-free collagen I gel control well would yield small IGFBP-2 and IGFBP-3 bands by ligand blot.

It was assumed that the intensity of the bands on the film was linearly related to IGFBP concentration. This was basically a qualitative assessment made by looking at the darkness of the bands. The system was not checked with known values to compare with each blot, and a range of linearity was not verified.

Another source of error was in the actual loading of the gel. Lyophilized samples were reconstituted with de-ionized water and an SDS buffer solution. After heating in a hot water bath, the entire pellet was not always dissolved. Returning the microcentrifuge tube to the hot water bath usually resolved this. This introduced some error in occasional lanes, but was not considered the major problem in terms of the analysis.

In retrospect, a method for better control bands on the ligand blot than that from the cell-free collagen I gel control would have been to use controls with a defined amount of IGFBP-2 and of IGFBP-3 instead of the unknown residual IGFBP after exposure of the cell-free gel to media with FBS. Another alternative for control bands would be the IGFBP-2 and IGFBP-3 bands resulting from cells stimulated with a known amount of IGF-I. These control bands would yield bands in the expected intensity range as the bands under investigation for comparison from blot to blot.

5.2 Part 2: Effect of 10% fetal bovine serum (FBS) on MAC-T cell attachment and growth

5.2.1 Introduction/Methods

MAC-T cells are grown and maintained with Dulbecco's modified Eagles medium (DMEM) + 10% fetal bovine serum (FBS). For the experiments to detect IGFBP secretion by MAC-T cells cultured on collagen I, cells were initially seeded with DMEM + 10% FBS and allowed to adhere over the next 24 h. After 24 h, the media was changed to plain DMEM, and the cells underwent serum starvation for the next 48 h. At that time IGF-I treatments were initiated according to experimental protocols, and conditioned media from the IGF-I stimulated cells were collected for IGFBP detection.

The question arose as to whether FBS acts in synergy with the collagen I on the cells. FBS contains a number of nutrients for the cells as well as extracellular matrix proteins such as fibronectin and vitronectin that could impact cell growth and therefore cell response to IGF-I and the subsequent amount of IGFBP-3 secretion. In addition, the possibility of differences in cell attachment to different ECM proteins brings up the question of whether collagen I surfaces have a different number of cells compared to tissue culture plastic surfaces, resulting in differences in IGFBP-3 detection due to a different cell number.

The DNA content of cells was measured from tissue culture plastic wells seeded with 200,000 MAC-T cells/well at 8, 24, 48, 72, and 96 h in the absence of FBS or in the presence of 10% FBS (Figure 5.2.1). In addition, DNA content of cells was measured for

a set of wells initially seeded in the absence of FBS for which 10% FBS was added after an 8 h attachment time. For all of these wells, media were removed after 24 h and replaced with DMEM without FBS. Cells were harvested 48 h later for DNA assay. Further, the DNA content of cells was determined from wells 48 h after seeding with MAC-T cells at a seeding density of 100,000 cells/well to tissue culture plastic wells, collagen I BIOCOAT wells, and collagen I gels in the absence of FBS and in the presence of 10% FBS (Figure 5.2.2). Media were changed to DMEM without FBS after 24 h.

5.2.2 Results

DNA content of cells seeded without FBS, without FBS for 8 h, and with 10% FBS was analyzed using the two-sample t-test assuming equal variance, $p < 0.05$, in Microsoft Excel. Cells seeded without FBS was less than for wells seeded with 10% FBS at 200,000 cells/well (Figure 5.2.1). The addition of 10% FBS after 8 h was not different from no FBS, as was to be expected, given the cells did not receive FBS for either grouping. Compared to cells seeded with 10 % FBS initially, both 8 h data points were significantly different from the 8 h point for seeding with 10% FBS. Within the no FBS grouping, only the 96 h data point was significant from control, and this was due to two high data points and two low data points not reflected in the mean and standard error. For the FBS after 8 h grouping, all subsequent data points (24, 48, 72, and 96 h) were significantly different from the initial 8 h data point. Likewise for the 24 h FBS grouping, all subsequent data points were significantly different from the 8 h initial data

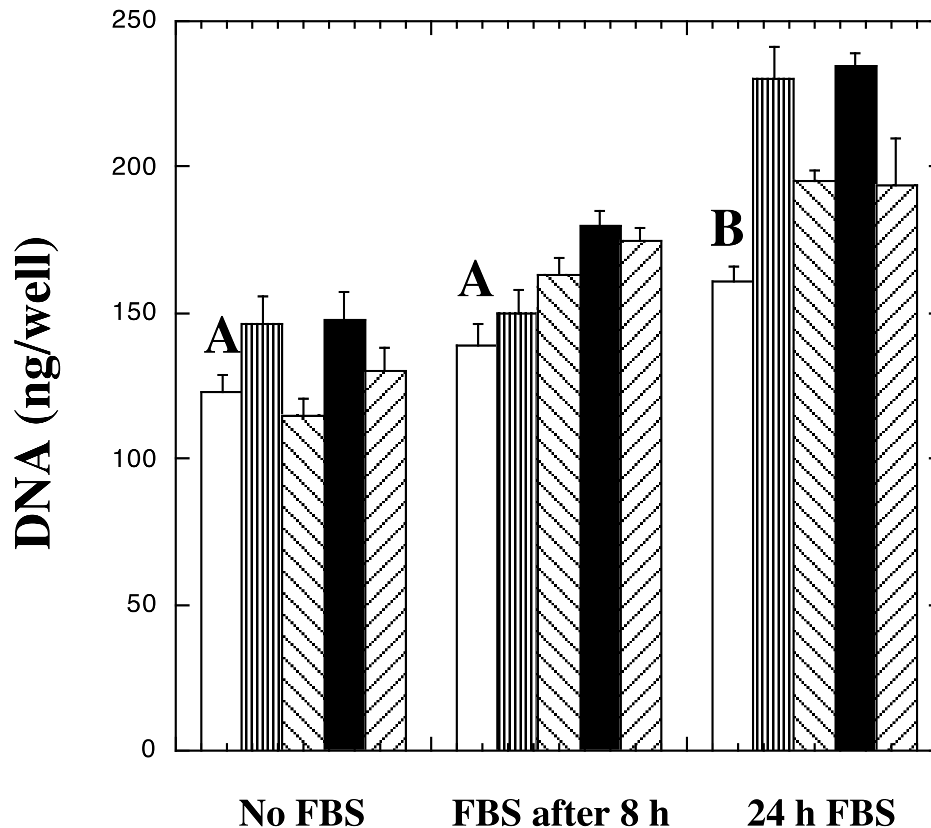

Figure 5.2.1. DNA content of plastic tissue culture wells seeded with 200,000 MAC-T cells/well. Three scenarios were investigated: no FBS, no FBS for the first 8 h followed by 16 h of 10% FBS, and 10% FBS for the first 24 h. Media was changed to plain DMEM after 24 h in all wells, and wells were harvested from each of the three scenarios at 8, 24, 48, 72, and 96 h. There are five columns in chronological order for each scenario, showing the DNA content at each time point (8, 24, 48, 72, and 96 h). Data for each column is from four separate wells measured in duplicate. Plot shows means ± SEM. Data is from one experiment. Two-sample t-tests assuming equal variances were performed in Excel with significance for $p < 0.05$. For the 8 h harvesting of cells data point, "No FBS" was not different from "FBS after 8 h" (each column marked as A). The "24 h FBS" (column marked as B) was significantly different for both "No FBS" and "FBS after 8 h" for the 8 h harvesting of cells. Compared to the 8 h point, only the 96 h point is significantly different from 8 h in the "No FBS" grouping (the data points were widely spread for 96 h.) In the "FBS after 8 h" grouping and in the "24 h FBS" grouping, the 24, 48, 72, and 96 h data points were all significantly different from the 8 h point.

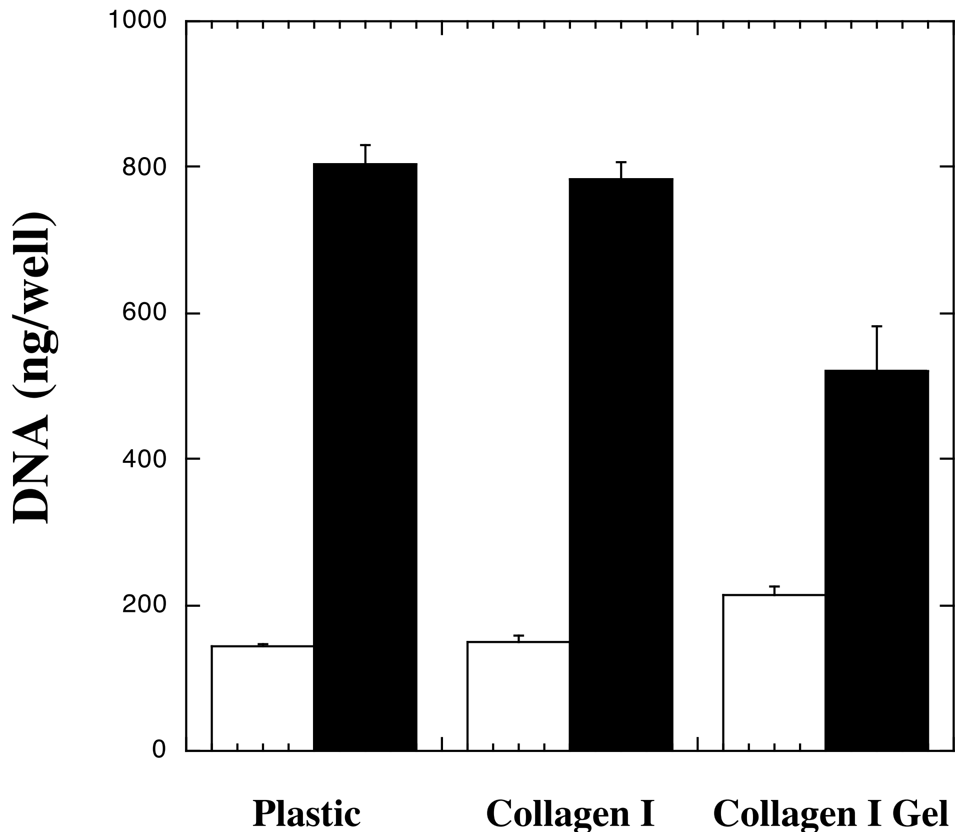

Figure 5.2.2. DNA content after 48 h from MAC-T cells seeded at 100,000 cells/well in the absence of FBS (open columns) and in the presence of 10% FBS (solid columns) on three different surfaces: tissue culture plastic, collagen I BIOCOAT, and collagen I gel. Data for each column is from four separate wells (plastic or collagen I BIOCOAT) or six separate wells (collagen I gel) and measured in duplicate. Plot shows means ± SEM. Data is from one experiment.

point. Figure 5.2.2 shows an increase in DNA with FBS for three surfaces (tissue culture plastic, collagen I BIOCOAT and collagen I gel).

5.2.3 Discussion

The presence of 10% FBS in the growth media for the first 24 h after cell seeding not only increases the initial attachment of MAC-T cells to tissue culture plastic, but it also contributes to the increase in downstream growth as measured by DNA content. Presence of 10% FBS for 16 h after the first 8 h of attachment also contributes to a downstream increase in DNA content. Cells that did not receive 10% FBS did attach to the surface, however, the DNA content of the wells remained stable over the next 96 h. In the next section (Chapter 5, Part 3), conditioned media from this experiment is analyzed for IGFBP-2 and IGFBP-3 content.

Comparison of the three different surfaces seeded with MAC-T cells either in the presence or absence of 10% FBS revealed that cells did attach in the absence of FBS. The data shown in Figure 5.2.2 suggests that the DNA content from 100,000 cells/well on a collagen I gel in the presence of 10% FBS is less than the DNA content for cells on either tissue culture plastic or collagen I BIOCOAT; however, this data is from a single experiment and pooled data from multiple experiments (Chapter 4, Figure 4.1) shows no difference in DNA content across the three different surface types for 50,000 cells/well and 200,000 cells/well. 10% FBS in the media shows significantly increased DNA content for all three surfaces. The conclusions from these studies are that FBS is not needed for the cells to attach to these surfaces and that FBS is required to elicit a downstream growth response as measured by an increase in DNA.

5.3 Part 3: Detection of IGFBP-2 and IGFBP-3 from conditioned media of MAC-T cells seeded without FBS for 24 h, without FBS for the first 8 h of 24 h, or with FBS the full first 24 h on tissue culture plastic

5.3.1 Introduction/Methods

As described in 5.2 Part 2, MAC-T cells seeded in the absence of FBS attached to tissue culture plastic, collagen I BIOCOAT, and collagen I gel surfaces. DNA assay of cells cultured on tissue culture plastic revealed that an increase in DNA content did not occur for cells in the absence of FBS over time. Conditioned media was collected from MAC-T cells cultured on each of the three surfaces under the conditions of no FBS or 10% FBS at the time of cell seeding. Further, 10% FBS was added to the media of some of the wells seeded without FBS after 8 h. Media was replaced with DMEM without FBS in all wells after the first 24 h. The cell conditioned media was collected from all wells after 48 h for processing to detect IGFBP-2 and IGFBP-3 by ligand blot (Appendix B).

5.3.2 Results

Neither IGFBP-2 nor IGFBP-3 were detected in the conditioned media of MAC-T cells cultured without FBS (Lanes 1 through 3, Figure 5.3.1). Cells that were initially seeded without FBS and received 10% FBS after 8 h secreted IGFBP-2 and IGFBP-3 (Lanes 4 through 6), as did cells seeded with 10% FBS (Lanes 7 through 9). Both tissue culture plastic (Lanes 4 and 7) and collagen I BIOCOAT (Lanes 5 and 8) surfaces had cells that produced minute amounts of IGFBP-3. Cells seeded to collagen I gel (Lanes 6 and 9) had strong bands of IGFBP-3. The IGFBP-3 band results for conditioned media from

cells seeded with 10% FBS on the three surfaces are consistent with other studies in this dissertation (Chapter 4 and Chapter 5, Part 1).

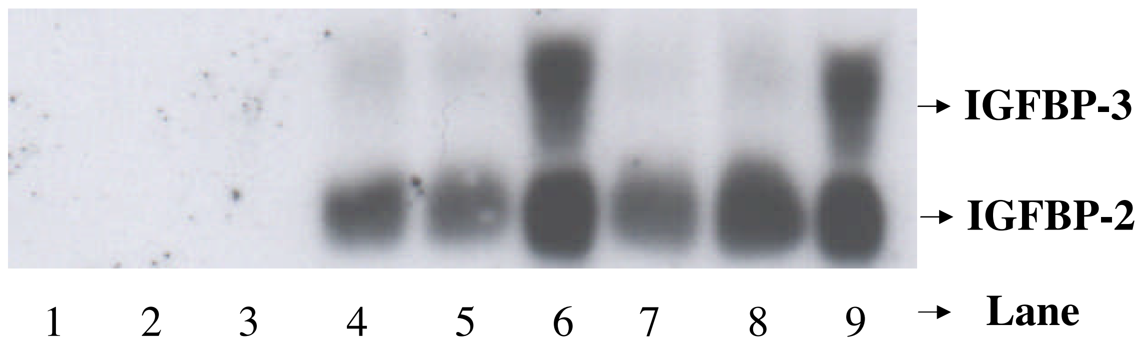

Figure 5.3.1. Ligand blot for conditioned media from MAC-T cells seeded without FBS for 24 h (lanes 1-3), from MAC-T cells seeded without FBS for the first 8 h of 24 h (lanes 4-6), and from MAC-T cells seeded with 10% FBS for the full first 24 h period (lanes 7-9). Lanes 1, 4, and 7 are from media from cells cultured on tissue culture plastic. Lanes 2, 5, and 8 are from media from cells cultured on collagen I BIOCOAT. Lanes 3, 6, and 9 are from media from cells cultured on collagen I gel. Samples from an independent experiment showed similar results.

5.3.3 Discussion

The ligand blot for IGFBP-2 and IGFBP-3 revealed that FBS is necessary for the secretion of these binding proteins from MAC-T cells cultured on tissue culture plastic, collagen I BIOCOAT, and collagen I gel. Initially, the possibility of seeding MAC-T cells in the absence of FBS to isolate the effect of collagen I from proteins present in serum was considered. Not only was an increase in DNA content (Chapter 5, Part 2) not

seen in the absence of FBS, but also no IGFBP was detectable after 48 of conditioning the media. The addition of FBS therefore remained critical in the investigations of cell response to collagen I in this dissertation, whether FBS was added from the start or after the first 8 h of cell adherence. Lanes 4 through 6 in Figure 5.3.1 show bands that are comparable to the bands in Lanes 7 through 8, demonstrating that the that the addition of serum after 8 h is sufficient for IGFBP secretion by the cells. Control bands (not shown here) from a cell-free collagen gel exposed to FBS were clearly visible on two separate blots that contained independent conditioned media samples from cells seeded without FBS, which showed no bands on either blot for the cells without FBS for 24 h..

5.4 Part 4: Polyacrylamide gels (PAGE) as alternate collagen I presentation

5.4.1 Introduction/Methods

MAC-T cells show different IGFBP secretion behaviors when cultured on collagen I BIOCOAT or on collagen I gel (Chapter 4). It is therefore conceivable that the presentation of collagen I in different spatial orientations to the cells might affect the ability of the cells to interact with and to respond to the collagen I molecules. It is not known how the cells cultured in a monolayer may deform to accommodate the growth surface of a collagen I BIOCOAT well compared to the growth surface of collagen I gel, for example. Similarly, it is not known how the different collagen I preparations may

affect the orientation of the collagen I in interaction with the cells and the cell surface receptors.

Dr. Joyce Wong of Boston University supplied prepared polyacrylamide gels (PAGE) for preliminary studies in this dissertation during which time she was investigating PAGE as a substrate to attach cells to measure the mechanical properties of the individual cells. The PAGE was deposited in the center of glass coverslips with the glass outer edges exposed. These coverslips were then placed in 6-well tissue culture plates for seeding. The gels were either plain or embedded with collagen I to increase cell adherence. To examine this matrix form for collagen I, MAC-T cells were seeded onto plain PAGE gels and collagen I embedded PAGE gels.

5.4.2 Results

Ideally, cells would have adhered to the PAGE preparations to provide a control with plain PAGE surfaces, and collagen I embedded in the PAGE preparations would show collagen I specific responses. The quantity of cells initially seeded was sufficient to obtain confluency on tissue culture plastic (>100,000 cells/cm^2). Unfortunately, only a few cells adhered to the PAGE gels when seeded, and after several days, the gels continued to only have a sparse number of adhered cells. Figure 5.4.1 shows light microscope photos of areas on the PAGE gels 24 h after seeding (1 x $10^{6)}$ MAC-T cells/well in the wells of 6-well tissue culture plates. The gels containing collagen I showed small clusters of cells in some areas, but with too much irregularity to be used in

a study to compare IGFBP secretion into the conditioned media. Cells adhered confluently to tissue culture plastic beyond the edges of the coverslips (Panel C) and to the glass edges of the coverslips (Panel E) on which the PAGE was deposited.

Figure 5.4.2 shows panels of light microscope photos after the media was replaced in the wells. The replacing of media dislodged cells that were not well adhered, and once again, cells adhered to the glass coverslip edges (Panel C) and to the tissue culture plastic, but not well enough to the PAGE material to achieve even near confluency. Some areas on the collagen embedded PAGE allowed clusters of cells to adhere (Panels E and F), but not with any uniformity across the entire PAGE surface.

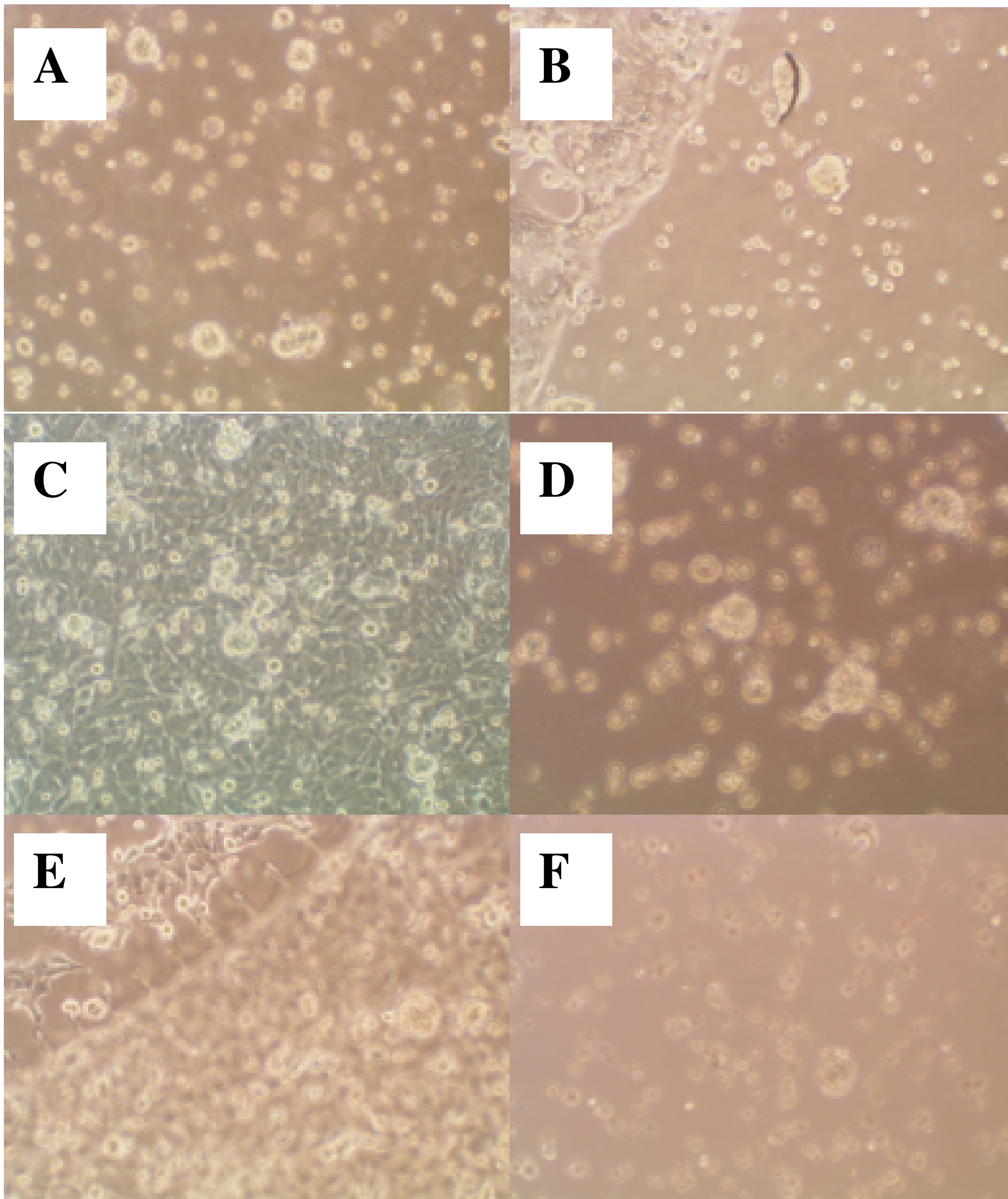

Figure 5.4.1. Light microscope photos of MAC-T cells on PAGE preparations 24 h after initial cell seeding (1,000,000 cells/well, 6-well plate). Panel A: plain PAGE, Panel B: edge of PAGE, cells can be seen on glass edge, Panel C: cells on plastic well surface, Panel D: cells in media above plain PAGE, Panel E: cells on edge of collagen I embedded PAGE, Panel F: cells near center of collagen I embedded PAGE.

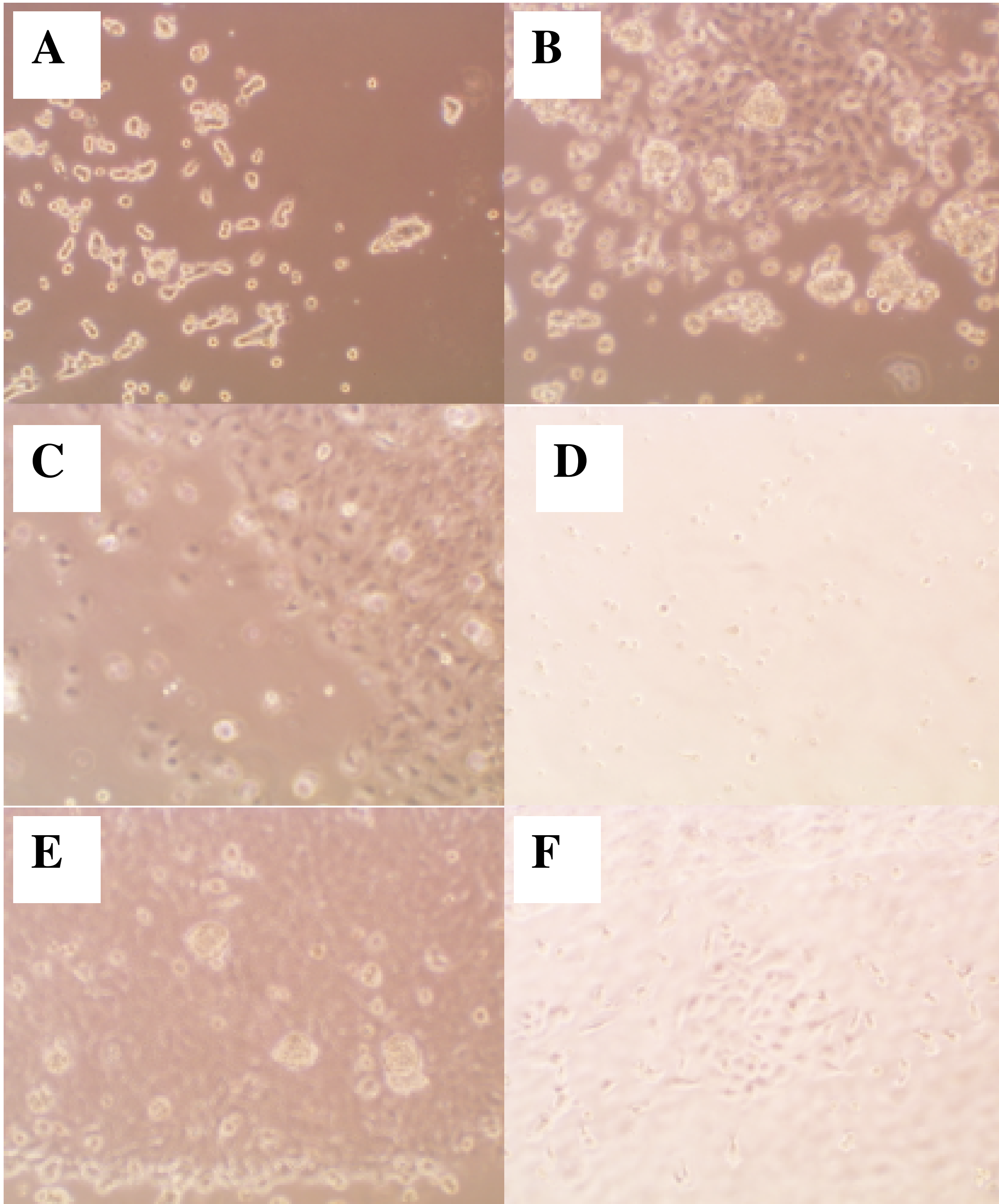

Figure 5.4.2. Light microscope photos of MAC-T cells adhered on PAGE preparations following media replacement 24 h after initial cell seeding (1,000,000 cells/well, 6-well plate). Panel A: cells on plain PAGE, Panel B: plain PAGE, cells can be seen on gel surface and floating in media, Panel C: cells on glass edge of plain PAGE, Panel D: cells in media above plain PAGE, Panel E: cluster of cells on collagen I embedded PAGE, Panel F: cluster of cells near glass edge of collagen I embedded PAGE.

5.4.3 Discussion

PAGE gels were found to adhere isolated cells, which perhaps would be useful to study mechanical properties of individual cells; however to detect IGFBP secretion effects from collagen I, uniform cell coverage was required to detect and to quantify IGFBP-2 and IGFBP-3 secretions for the purpose of comparison with other substrates. MAC-T cells did not adhere well to the PAGE material, and we reasoned that there were not enough cells present to detect IGFBP secretion, let alone quantify a collagen I effect; however, the possibility of embedding this material with ECM proteins remains promising. Cells adhered in clusters on collagen I embedded gels, and investigation of this material with more uniform and dense coverage of collagen I and other ECM proteins may provide an alternate means of presenting ECM proteins to cells to study resultant cell response both in terms of cell morphology and in terms of cell-secreted proteins.

5.5 Part 5: IGFBP-2 and IGFBP-3 secretion by cells on a porous polycarbonate membrane

5.5.1 Introduction/Methods

MAC-T cells were cultured on the polycarbonate membrane surface of transwells (12 mm, pore size 3.0 μm) for stimulation of the cells by IGF-I on the Cytosensor microphysiometer (Chapter 2). The same transwells in a 12-well tissue culture plate provided a system for investigating the effects of a co-culture of two different cell lines. This system was utilized to detect changes in patterns of IGFBP secretion into the media, presumably via a cross-talk mechanism since cells communicate via proteins or other

substances secreted into the extracellular space. The two cell lines would be physically isolated, yet share the same growth media due to the porous membrane of the transwell. The plastic sides of the transwell are solid, and media and dissolved components can only pass through the porous membrane of the transwell, as cells allow. Figure 5.5.1 shows two configurations where one cell line can be cultured in the transwell and a second cell line can be cultured in the well holding the transwell in a tissue culture plate. Media samples were collected from outside the transwell and from within the transwell for different configurations of MAC-T and SV40-IGF-I cell cultures. These samples were then analyzed for IGFBP-2 and IGFBP-3 content using the ligand blot procedure (Appendix B). In addition to co-culturing the two cells lines, controls were performed for each line in which only one cell line was seeded to the first surface, and the second surface in the transwell/tissue culture well system remained free of cells. Cell seeding density was 270,000 cells per transwell or per tissue culture well to achieve confluent monolayers of cells after 24 h incubation with Dulbecco's modified Eagles medium (DMEM) +10% FBS. Media was then removed and replaced with serum-free media to serum starve the cells for 72 h. After the serum starvation period, the transwells were placed into the corresponding wells for co-culture. Conditioned media were collected from the co-cultures after 48 h incubation. Of particular interest was the IGFBP-2 and IGFBP-3 content in media for MAC-T cells cultured on the polycarbonate membrane of the transwell in the absence of the second cell line.

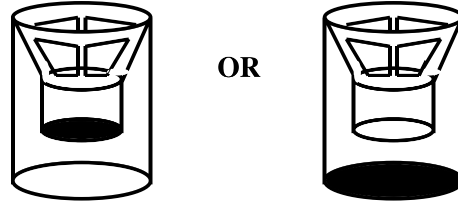

Figure 5.5.1. Each view shows a transwell placed in the cylindrical well of a 12-well tissue culture plate. Cells were seeded on the polycarbonate membrane of the transwell and on the bottom of the well in the 12- well plate. Shaded areas represent cell monolayers. Growth medium was added both into the transwell and into the outer well.

5.5.2 Results

Figure 5.5.2 shows a ligand blot with bands of IGFBP-3 and of IGFBP-2 from media collected from the transwell/tissue culture well systems of cells seeded either with the MAC-T cell line or the autocrine SV40-IGF-I cell line. Lanes 1 through 3 show IGFBP bands from media collected from SV40-IGF-I cells. Lane 1 shows bands for conditioned media collected from a tissue culture well with SV40-IGF-I cells. Lanes 2 and 3 show media collected from a co-culture system where the outer well (2) is cell-free and SV40-IGF-I cells are cultured on the transwell membrane (3). Strong bands are shown for both IGFBP-3 and IGFBP-2 from media in the transwell, and IGFBP-3 and IGFBP-2 were secreted across the porous polycarbonate membrane into the outer well.

MAC-T cells are represented in lanes 4 through 6 of Figure 5.5.2. Lane 4 shows a strong band for IGFBP-2 and a faint band for IGFBP-3 from media collected from a well with

MAC-T cells. In lanes 5 and 6, bands from media from the co-culture system with a cell-free well (5) and with MAC-T cells cultured on the transwell polycarbonate membrane (6). Comparison of these three lanes shows there is a profound increase in IGFBP-3 secretion by MAC-T cells on the porous polycarbonate membrane of the transwell as compared to MAC-T cells cultured on tissue culture plastic. Further, the IGFBP-3 appears predominantly on the transwell side of the co-culture system.

Figure 5.5.3 shows the ligand blot to detect IGFBP bands for the co-culture systems with MAC-T and SV40-IGF-I cells. Lanes 1 and 2 show the bands for IGFBP-3 and IGFBP-2 for the system with MAC-T cells on the transwell membrane surface and SV40-IGF-I cells in the tissue culture well. A strong signal for both IGFBP-3 and IGFBP-2 from media collected from the transwell (2) with MAC-T cells can be seen, as is the case in the previous figure. IGFBP-3 and IGFBP-2 bands on the SV40-IGF-I well (1) side are visible, but not nearly as pronounced. Lane 4 shows the strong bands for SV40-IGF-I cultured on transwells, with secretion to the cell-free well (3). Lanes 5 and 6 show very strong bands for IGFBP-3 and IGFBP-2 on both sides in the co-culture system with MAC-T cells in the transwell and SV40-IGF-I cells in the outer well. Lanes 7 and 8 illustrate once again a faint band for IGFBP-3 and a strong band for IGFBP-2 for MAC-T cells cultured on tissue culture plastic with a cell-free transwell.

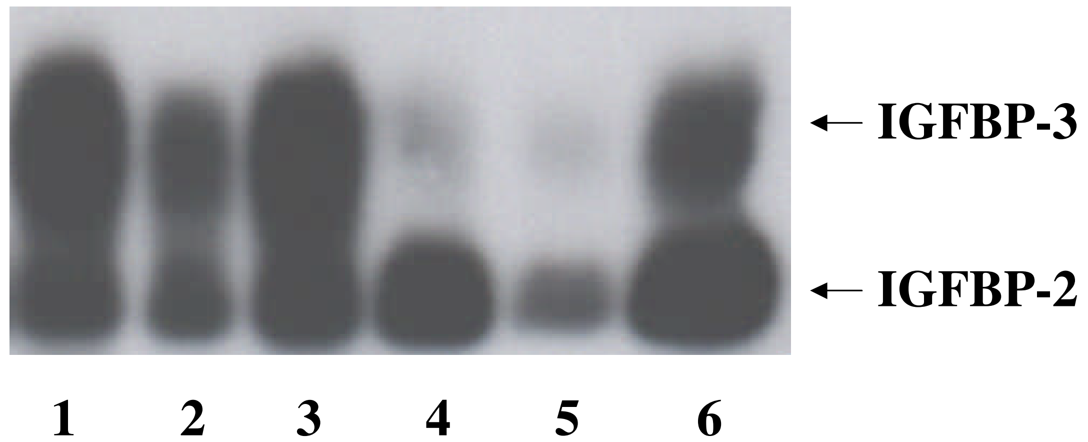

Figure 5.5.2. Ligand blot to detect IGFBP-3 and IGFBP-2 from transwell system for MAC-T and SV40-IGF-I cells. Lane 1 shows the bands from SV40-IGF-I cells cultured in a well without the transwell. Lanes 2 and 3 show bands from the outer well (2) and from the inner transwell (3) for the SV40-IGF-I control of cells cultured on the transwell membrane only. Lane 4 shows the bands from MAC-T cells cultured in a well without the transwell. Lanes 5 and 6 show bands from the outer well (5) and from the inner transwell (6) for the MAC-T control of cells cultured on the transwell membrane only. Results are from a single experiment.

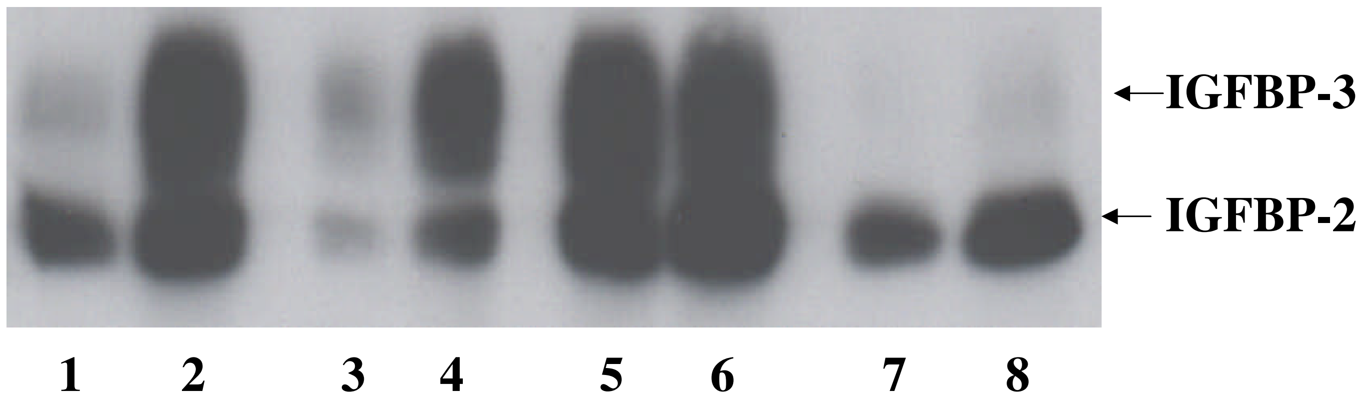

Figure 5.5.3. Ligand blot to detect IGFBP-3 and IGFBP-2 from transwell system for co-cultures of MAC-T and SV40-IGF-I cells. Lanes 1 and 2 show bands from the inner transwell (1) and from the outer well (2) for the co-culture of MAC-T cells on the transwell membrane and SV40-IGF-I cells in the outer tissue culture well. Lanes 3 and 4 show bands from the inner cell-free transwell (3) and from the outer well (4) for the SV40-IGF-I control of cells cultured in the outer well only. Lanes 5 and 6 show bands from the inner transwell (5) and from the outer well (6) for the co-culture of SV40-IGF-I cells on the transwell membrane and MAC-T cells in the outer tissue culture well. Lanes 7 and 8 show bands from the inner cell-free transwell (7) and from the outer well (8) for the MAC-T control of cells cultured in the outer well only. Results are from a single experiment.

5.5.3 Discussion

The increase in cell-secreted IGFBP-3 detected from the transwell of MAC-T cells is very interesting, because MAC-T cells secrete very minute amounts of IGFBP-3 when cultured on tissue culture plastic in the absence of IGF-I. Comparison of known secretion patterns of MAC-T cells cultured on tissue culture plastic with the bands in lanes 7 and 8 in Figure 5.5.3 where MAC-T cells were cultured on the tissue culture well and the transwell was cell-free, barely discernible shadows for IGFBP-3 bands are

visible, which were expected. The very pronounced IGFBP-3 band shown in lane 6 in Figure 5.5.2 shows that IGFBP-3 was secreted to the apical side of the MAC-T cells culture on the transwell. The very clear increase in IGFBP-3 secretion for MAC-T cells on the polycarbonate membrane surface of the transwell warrants further investigation. The interaction of the cells with the surface may result in changes in cell morphology to stimulate new IGFBP secretion patterns, or the polycarbonate membrane surface may interact with cell surface integrins in spatial orientations that stimulate IGFBP secretion.

Also of interest in these preliminary experiments is the effect of the co-culture of the IGF-I autocrine SV40-IGF-I cell line with the MAC-T cell line. IGF-I autocrine cells produced IGFBP-3 when cultured both on the transwell polycarbonate membrane and on tissue culture plastic. The presence of MAC-T cells on the transwell surface resulted in less IGFBP detected in media from the outer well with SV40-IGF-I cells. This also warrants further investigation. MAC-T cells on the transwell surface had a strong IGFBP-3 signal on the apical side of the cells. This may be due to IGFBP-3 being secreted preferentially to the apical side of the cells, or the presence of proteases on the basolateral side of the cells. In this case, the presence of proteases or some inhibitor of IGFBP on the basolateral side of the MAC-T cells could also account for the decreased IGFBP signal from SV40-IGF-I cells in the outer well.

5.6 Part 6: Other extracellular matrix proteins: laminin and fibronectin BIOCOAT plates

5.6.1 Introduction/Methods

MAC-T cells cultured on the collagen I BIOCOAT (Becton Dickinson) did not yield the increases in IGFBP-3 secretion and decreases in IGFBP-2 secretion observed from MAC-T cells cultured on a collagen I gel (Chapter 4, Chapter 5, Part 1). However, it has been noted in other cell lines that phenotypic expression of cells is impacted by different extracellular matrix (ECM) proteins (Chapter 1). IGFBP secretion was measured for MAC-T cells cultured on additional ECM proteins in the commercially available BIOCOAT plates: human fibronectin and laminin. Wells in 24-well plates with the different BIOCOAT surfaces were seeded with 200,000 MAC-T cells per well, and cell-secreted IGFBP-2 and IGFBP-3 were detected using the procedure described in Appendix B. DNA content of each well was determined using the DNA assay procedure described in Chapter 3 Materials and Methods. Additionally, IGF-I (100ng/mL) was added to some of the wells to detect IGF-I stimulated increases in IGFBP-3 secretion, since it is known to stimulate IGFBP-3 secretion in MAC-T cells cultured on tissue culture plastic.

5.6.2 Results

Figure 5.6.1 shows the ligand blot from the conditioned media of MAC-T cells cultured on tissue culture plastic or on BIOCOAT plates with collagen I, laminin, and fibronectin. Figure 5.6.2 shows the relative intensity of bands for IGFBP-3. IGFBP-3 bands on a ligand blot (Appendix B) were measured with densitometer software. The results shown

are from a single preliminary experiment, and media were pooled from three wells for each surface. IGFBP-3 content in the media increases for IGF-I stimulated cells cultured on tissue culture plastic, collagen I, and laminin. (The IGFBP-3 signal in the presence of IGF-I is essentially doubled on the well-exposed ligand blot.)

The intensity of IGFBP-2 bands from ligand blot for the four surfaces is shown in Figure 5.6.3. IGFBP-2 detection on the different BIOCOAT surfaces investigated did not differ from tissue culture plastic. The addition of IGF-I was not investigated with cells on the fibronectin.

DNA content of cells per well on the four surfaces is shown in Figure 5.6.4. Collagen I BIOCOAT did not differ from tissue culture plastic, consistent with results shown in Chapter 4. Laminin and fibronectin showed less DNA, which is not surprising since cell adherence can be expected to vary on different coatings *(5)*. All surfaces showed increased DNA in the presence of IGF-I stimulation.

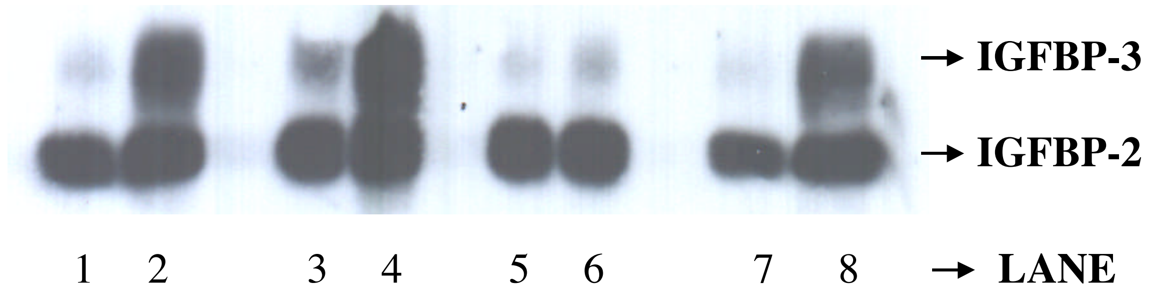

Figure 5.6.1. Ligand blot for conditioned media from MAC-T cells cultured on tissue culture plastic (lanes 1 and 2), collagen I BIOCOAT (lanes 3 and 4), fibronectin BIOCOAT (lanes 5 and 6), and laminin BIOCOAT (lanes 7 and 8). The first lane for each surface type is from media without FBS. The second lane is from media with 100 ng/mL IGF-I with the exception of the fibronectin lane 6, which was not treated with IGF-I and is a replicate of lane 5.

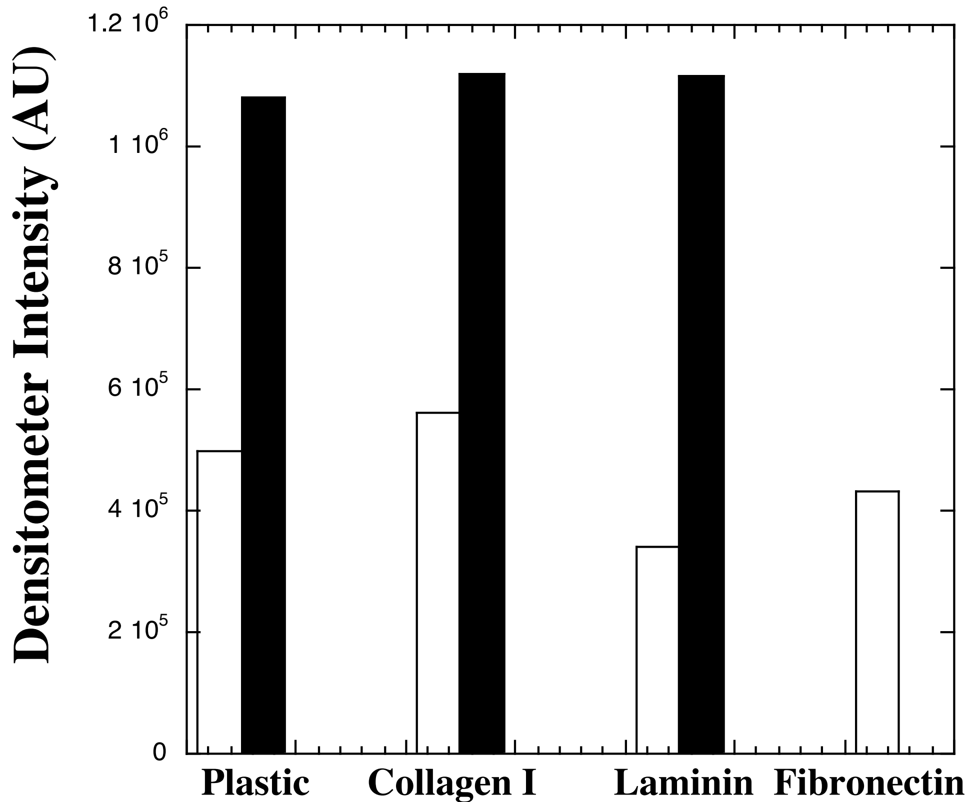

Figure 5.6.2. IGFBP-3 band intensities from ligand blot as measured by densitometry for tissue culture plastic, collagen I BIOCOAT, laminin BIOCOAT, and fibronectin BIOCOAT. Open columns are secreted IGFBP-3 band intensities from MAC-T cells without IGF-I; solid columns are secreted IGFBP-3 band intensities from IGF-I (100ng/mL) stimulated MAC-T cells. Each bar is from pooled media from three separate wells from a single experiment.

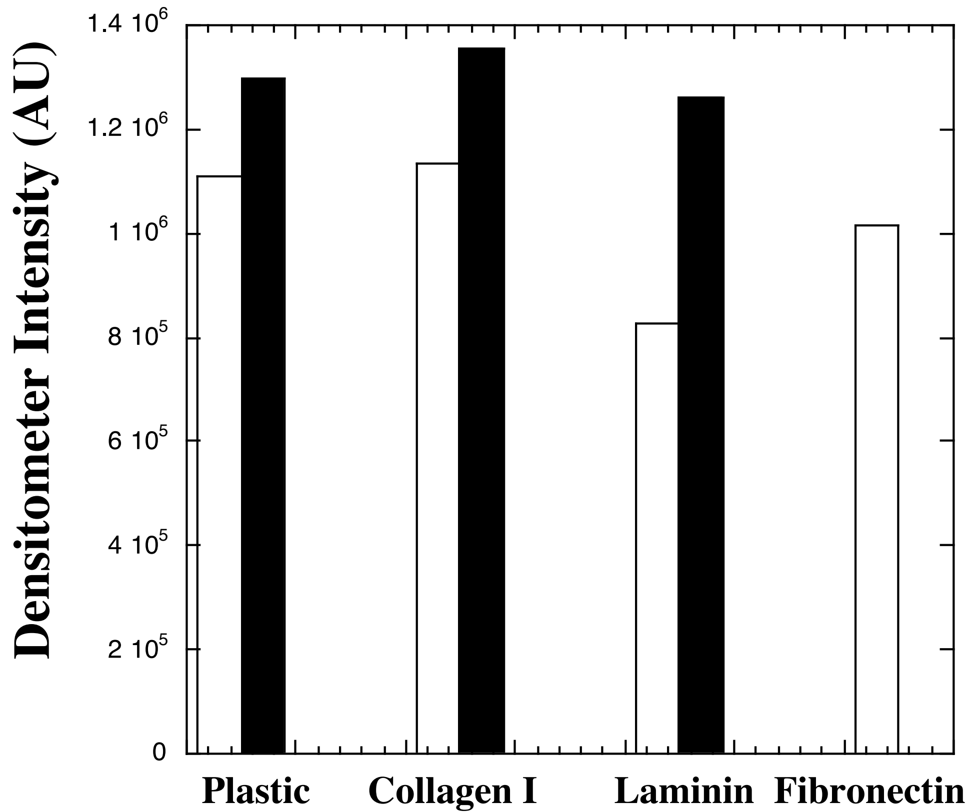

Figure 5.6.3. IGFBP-2 band intensities from ligand blot as measured by densitometry for tissue culture plastic, collagen I BIOCOAT, laminin BIOCOAT, and fibronectin BIOCOAT. Open columns are secreted IGFBP-2 band intensities from MAC-T cells without IGF-I; solid columns are secreted IGFBP-2 band intensities from IGF-I (100ng/mL) stimulated MAC-T cells. Each bar is from pooled media from three separate wells from a single experiment.

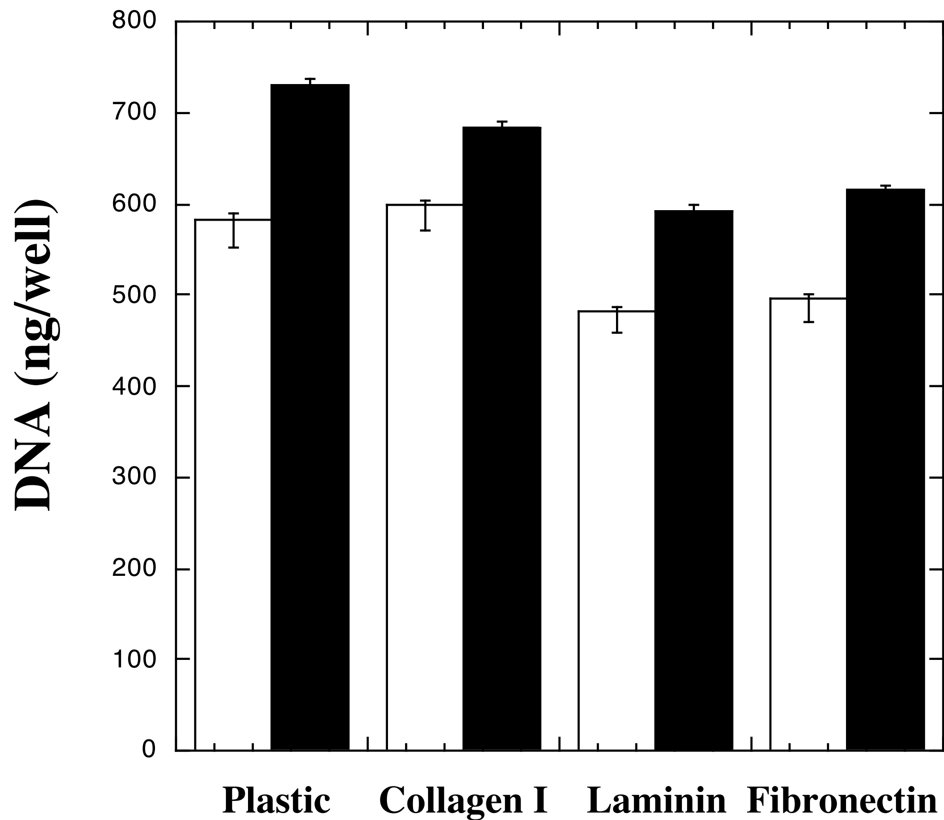

Figure 5.6.4. DNA content per well for MAC-T cells cultured on tissue culture plastic, collagen I BIOCOAT, laminin BIOCOAT, and fibronectin BIOCOAT. Open columns are from MAC-T cells without IGF-I; solid columns are from IGF-I (100ng/mL) stimulated MAC-T cells. Measurements were made in duplicate for each of three wells. Plot shows means ± SEM (three wells per sample).

5.6.3 Discussion

The BIOCOAT plates with the different ECM proteins (collagen I, laminin, fibronectin) did not reveal differences in IGFBP secretion by the MAC-T cells as compared to tissue culture plastic. There were slight differences in number of cells adhered to the different

surfaces, which was not surprising given that other investigators have noted differences in cell adhesion on different ECM proteins *(5)*. Although this set of experiments did not reveal an effect on IGFBP secretion by the MAC-T cells as a result of the ECM proteins, further investigations with surfaces that have the ability to change cell morphology or that can create otherwise spatially unique surfaces to interact with the cells may reveal additional possibilities in cell responses to different ECM proteins.

5.7 References

1. Richards, J., Larson, L., Yang, J., Guzman, R., Tomooka, Y., Osborn, R., Imagawa, W., and Nandi, S. (1983). Method for culturing mammary epithelial cells in a rat tail collagen gel matrix. *J. Tissue Cult. Methods.* **8**, 31-36.
2. Birchmeier, C., Meyer, D., and Riethmacher, D. (1995). Factors controlling growth, motility, and morphogenesis of normal and malignant epithelial cells. *Intl. Rev. Cytology.* **160**, 221-266.
3. Huynh, H.T, Robitaille, G., and Turner, J.D., (1991). Establishment of bovine mammary epithelial cells (MAC-T): an in vitro model for bovine lactation. *Exp. Cell Res.* **197**, 191-199.
4. Freshney, R.I. (1987). Culture of Animal Cells (2nd edition). Alan R. Liss, Inc. New York.
5. McCaig, C., Perks, C.M., and Holly, J.M.P. (2002). Intrinsic actions of IGFBP-3 and IGFBP-5 on Hs578T breast cancer epithelial cells: inhibition or accentuation of attachment and survival is dependent upon the presence of fibronectin. *Journal of Cell Sci.* **115**, 4293-4303.

CHAPTER 6

Summary of results and recommendations for further research

Investigation of the effect of autocrine IGF-I and of collagen I gel on a bovine mammary epithelial cell line revealed that cell response can be modulated by modifications in the availability of these two proteins which are naturally found in the extracellular environment. The effects of autocrine IGF-I and of collagen I gel were investigated separately.

The bovine mammary epithelial cell line (MAC-T) transfected to produce autocrine IGF-I was found to have an alteration in the IGF-I signaling pathway in that the cells retained sensitivity to exogenous IGF-I, but this sensitivity did not result in downstream cell proliferation above the baseline response (Chapter 3). The autocrine IGF-I cells proliferated to higher densities in culture as compared to the parental MAC-T cells, but the de-coupling of the exogenous IGF-I signal from downstream proliferation demonstrated that the autocrine IGF-I in the cell results in self-limiting proliferation. In other words, additional proliferation did not result with an increase in exogenous IGF-I stimulated IGF-IR signaling as measured by extracellular acidification. Where the break in the IGF-I signaling pathway occurs in the IGF-I autocrine cell has not yet been determined. Further investigations in the signaling pathway comparing MAC-T and IGF-I autocrine cells can elucidate points in the pathway that are critical in the IGF-I stimulated proliferation of mammary epithelial cells. This de-coupling of the exogenous IGF-I signal from downstream proliferation has relevance in IGF-I stimulated cancerous

cells, as it may identify points in the signaling pathway that can be targeted to slow or to prevent the proliferation of cancerous cells.

Observations have been made by a number of investigators on how the phenotypic expression of cells is modified by extracellular matrix proteins (ECMs) *(1,2)*. In our preliminary studies with BIOCOAT (Becton Dickinson) preparations of collagen I, collagen IV, fibronectin, and laminin, differences in IGFBP secretion patterns were not found (Chapter 5, Part 6). However, it was found that cells cultured on a collagen I gel (300 μL/well rat-tail, in-house preparation) had an increased amount of IGFBP-3 and a decreased amount of IGFBP-2 in conditioned media compared to MAC-T cells cultured on tissue culture treated plastic (Chapter 4).

This observation is exciting although the cause remains unknown. The surfaces of the different substrates were not characterized, and it is conceivable that how the cells interact with the collagen I gel may impact the binding protein secretion patterns of the cells. Cell shape *(3)* has been shown to be critical in apoptosis, a PI-3 pathway programmed death of cells *(2)*. How the cells interact spatially with the surface as well as how the surface might impact the shape of the interacting cells is of interest in subsequent cell growth response and in the related changes in protein secretions. This has implications in agriculture for increasing mammary cells in milk-producing animals as well as in medical applications for the reduction of cell growth in cancerous cell proliferation. Agriculturally, genetic modifications of the animal may result in increased mammary epithelial cell growth by modification of ECM secretions in the extracellular

environment. Medically, it may be that the spatial presentation, and not just the presence, of different ECM proteins may be crucial in the phenotypic expression of the cells. A topical, local introduction of naturally occurring ECM proteins during surgery or by injection may be a possibility to inhibit cancer growth or reoccurrence in conjunction with other treatments if shown to be effective in slowing or inhibiting cell growth when introduced at higher than normal concentrations in spatially effective preparations.

6.1 Effect of autocrine IGF-I on MAC-T cell response

6.1.1 Summary of results in this dissertation for autocrine IGF-I

1.) Both MAC-T cells and MAC-T cells transfected to secrete IGF-I responded to exogenous IGF-I with dose-dependent extracellular acidification, indicative of IGF-I receptor stimulation (Chapter 2, Figure 2 and Chapter 3, Figure 2).

2.) The extracellular acidification response to IGF-I was reduced for both cell lines with the addition of exogenous IGFBP-3 to the media with IGF-I (Chapter 2, Figure 3 and Chapter 3, Section 3.3 Results).

3.) IGF-I autocrine cells demonstrated protein synthesis in response to exogenous IGF-I, as did the parental MAC-T cells (Chapter 3, Figure 3).

4.) IGF-IR stimulation by exogenous IGF-I was un-coupled from downstream proliferation in the autocine cell line. Autocrine cells did not respond to exogenous IGF-I with increased proliferation as measured by [^{3}H]thymidine incorporation (Chapter 3, Figure 4). Parental cells incorporated [^{3}H]thymidine in an IGF-I dose-dependent manner (Chapter 2, Figure 4).

5.) Lack of proliferation in response to exogenous IGF-I by the autocrine cells could not be accounted for by stagnant conditions as compared to the flow conditions under which the extracellular acidification was measured (Chapter 3, Figure 7).

6.) Lack of proliferation to exogenous IGF-I by the autocrine cells could not be accounted for by the growth surface. Neither tissue culture plastic nor polycarbonate membrane yielded a proliferation response in autocrine cells from exogenous IGF-I as measured by [^{3}H]thymidine incorporation (Chapter 3, Figures 4 and 6).

6.1.2 Recommendations for future work with IGF-I signaling

1.) Continue to investigate the IGF-IR signaling pathway in MAC-T cells and in the IGF-I autocrine cells for known points of phosphorylation in order to elucidate where de-coupling of the IGF-IR signal from downstream proliferation occurs in the IGF-I autocrine cells. This will be helpful in determining potential points for medical interruption of the IGF-IR signaling pathway in IGF-I stimulated cancerous cells.

Commercially available kits for the IGF-IR signaling pathway may be utilized to detect which points of phosphorylation in the pathway become activated for both cell lines.

2.) Investigate the media of cultured cells for the presence of proteases, which may break down detectable binding proteins. This can be done by adding known amounts of IGFBPs to cultured media and detecting residual IGFBP over the course of time by ligand blot. The breaking down of binding proteins in the media could account for the discrepancy of the amounts of detected IGFBP mRNA in the cells with the amounts of detectable IGFBP in the media (Chapter 4).

6.2 Effect of collagen I gel substrate on MAC-T cell response

6.2.1 Summary of results in this dissertation for collagen I gel

1.) Plating of cells on a collagen I gel increased the secretion of IGFBP-3 into the media by MAC-T cells (Chapter 4, Figure 1).

2.) Plating of cells on a collagen I gel decreased the secretion of IGFBP-2 into the media by MAC-T cells (Chapter 4, Figure 1).

3.) Plating of cells on collagen I BIOCOAT plates did not yield the change in IGFBP-2 and IGFBP-3 secretion observed on the collagen I gel (Chapter 4, Figure 1).

4.) IGFBP-2 mRNA decreased in the presence of 100 ng/mL IGF-I for MAC-T cells on tissue culture plastic, collagen I BIOCOAT, and collagen I gel (Chapter 4, Figure 5).

5.) IGF-IR phosphorylation occurred in MAC-T cells only in the presence of IGF-I on tissue culture plastic, collagen I BIOCOAT, and collagen I gel (Chapter 4, Figure 4).

6.) DNA content was comparable for MAC-T cells cultured on tissue culture plastic, collagen I BIOCOAT, and collagen I gel. DNA content increased with IGF-I on all three surfaces (Chapter 4, Figure 3).

7.) Residual IGF-I content in the media of MAC-T cells cultured on tissue culture plastic, collagen I BIOCOAT, and collagen I gel was comparable on all three surfaces, indicative of similar rates of IGF-I incorporation and degradation (Chapter 4, Table 1) .

8.) Preliminary studies did not reveal differences in IGFBP-2 and IGFBP-3 secretion by MAC-T cells on BIOCOAT preparations of collagen I, collagen IV, fibronectin, laminin, or poly-D-lysine (Chapter 5, Figures 5.6.1 and 5.6.2).

9.) Preliminary studies found similar increases in IGFBP-3 in conditioned media from cells plated on collagen I, collagen IV, laminin, and poly-D-lysine BIOCOAT surfaces in response to exogenous IGF-I (100 ng/mL) (Chapter 5, Figure 5.6.1). Fibronectin BIOCOAT was not tested with IGF-I.

10.) Preliminary studies showed that the culturing of MAC-T cells on a polycarbonate membrane increased IGFBP-3 secretion into the media by these cells (Chapter 5, Figure 5.5.2).

6.2.2 Recommendations for future work with ECM proteins

1.) Investigate the IGFBP secretion by MAC-T cells plated on collagen I and other common ECM proteins such as fibronectin, collagen IV, and laminin of different gel layer thickness. In a preliminary study, no significant change was found in the amount of IGFBP-2 or –3 secreted by MAC-T cells adhered to collagen I, fibronectin, collagen IV, or laminin BIOCOATS (thin coatings on plastic). However, an effect was seen with the collagen I gel, and investigation of other ECM proteins in different substrate forms may show an ECM effect in cell phenotypic expression and morphology. As an initial investigation, collagen I gels could be prepared to determine if IGFBP secretion is dependent on the gel thickness. With this information, gels of the other ECM proteins could be prepared with gel thickness corresponding to the collagen I experiments, and the resulting IGFBP secretion as measured by ligand blot analysis for each ECM and each gel thickness could be compared to the collagen I gel baseline experiments. This would be useful in anticipating if ECM layer depth would be important to IGFBP production in a living animal system.

2.) Characterize the cell morphology required for changes in IGFBP secretion patterns. For the studies described in this dissertation, how the surface substrate might impact the

cell morphology and how the cells might interact spatially with the surface were not investigated. This could be further investigated by preparing surfaces with ECM proteins in non-continuous patterns as has previously been done in a different application *(3)*. Cell morphology could be observed and measured using microscopy, and IGFBP secretion could be measured by ligand blot analysis of the cell cultured media.

3.) Continue to investigate different matrix presentations of collagen I to determine if cells will interact with the ECM protein in a particular orientation or form to stimulate IGFBP-3 (or IGFBP-2) production. Dr. Joyce Wong of Boston University is researching how single cells are oriented on a PAGE gel (polyacrylamide gel) with added ECM proteins. A sufficient number of cells did not adhere to this experimental material with collagen I to collect measurable amounts of cell secreted IGFBP-2 and IGFBP-3, but this could be re-investigated with increased collagen deposition on the gel. Collaboration with her to compare single cell orientation measurements would be useful if measurable amounts of IGFBP can be collected. This would validate the dependency of cell spatial orientation to ECM induced IGFBP production.

4.) Conduct IGF-I receptor binding studies to see if the collagen I gel impacts the number of IGF-I receptors on the cell surface. This is important in determining if increased IGFBP-3 production by cells on a collagen I gel is stimulated via the IGF-I stimulation of an increased number of IGF-I receptors. The study can be extended to cells plated on other ECM proteins as IGFBP-3 secretion results are collected.

5.) Measure IGFBP-2 and IGFBP-3 adhered or bound in the growth substrate. The ligand blot measurements in the studies in this dissertation only took into account binding protein secreted into the culture media, and the measurements of the IGFBPs did not correspond to the levels of cell RNA for the IGFBPs (Chapter 4). The collagen I gel may have created a reservoir for cell-secreted binding proteins, which were not measured. IGFBP-2 and IGFBP-3 content in the media of cell-free systems processed with FBS containing media for background signals was measured, but this did not take into account additional IGFBP that may have collected in the gel for gels with cells.

6.) Continue to investigate the IGF-IR signaling pathway for cells grown on different ECM proteins and compare the intensity of phosphorylation for known points in the pathway for cells adhered on the different matrices to detect if pathway changes occur as a result of ECM protein stimulation. This can verify if IGFBP-3 production by MAC-T cells plated on collagen I gel is directly related to stimulation of the same points in the IGF-I signaling pathway as for MAC-T cells plated on tissue culture plastic. It can also elucidate if increased IGFBP-3 secretion is directly related to increased phosphorylation at specific points in the pathway.

7.) Investigate serum-free systems (vitronectin and fibronectin-free systems) for ECM modulated IGFBP production. Since vitronectin and fibronectin interact via the same receptors as the ECM proteins of interest, they may confound the IGFBP results if stimulation of IGFBP production occurs via receptors that also bind these proteins. A lack of activity in the absence of vitronectin and fibronectin would not necessarily negate

the effects of the ECM proteins under investigation, as it is conceivable that there may be a synergistic effect between those ECM proteins and vitronectin or fibronectin.

8.) Investigate 3-dimensional tissue systems for ECM protein-modulated IGFBP production. This requires a more complex structure to be designed based on results from the 2-dimensional ECM surface studies, and it would be the next step in applying learned ECM effects to an animal system. It could involve plating cells to different layers of different ECM proteins, and it would require complex experimental analyses to observe and to take into account the effects of cell cross-talk as a result of stimulation by the different proteins at variable concentrations and/or configurations. The long-term benefit of developing such a model would be the ability to make some quantitative predictions of the effects of different variables in the growth substrata on cell growth and differentiation. This could be extended to predictions to apply to animal models either to inhibit or stimulate cell growth.

9.) Investigate the effect of ECM proteins on SV40-IGF-I cells to determine if IGF-I transfection in cells impacts the ECM effect on the IGF-I signaling pathway, including the IGFBP-3 secretion response. This could possibly elucidate if and where in the IGF-I signaling pathway ECM protein induced IGFBP-3 production occurs.

10.) Further investigate ligand blot measurements to see if this system can be tweaked to achieve the precision needed for quantitative comparison of binding protein from one blot to the next. To start, a blot can be made with known ligand concentrations to determine

the viability of this method and to confirm a linear relationship between exposure time and band intensity. Instead of using a background cell-free collagen I gel, which detects residual IGFBP-2 and IGFBP-3 left behind by FBS, a control either of media from MAC-T cells stimulated with IGF-I might be used or of previously quantified amounts of IGFBP-2 and IGFBP-3. Also investigate if alternate methods can be used to determine IGFBP concentrations.

The work in this dissertation has focused on selected modifications in the cellular environment specific to components of the IGF-I signaling pathway in cultured mammary epithelial cells. It has been shown that by relatively small modifications in the amount of or in the presentation of the naturally occurring proteins IGF-I and collagen I, the response of cells can be impacted. For IGF-I, it has been found that autocrine IGF-I can turn off the ability of cells to respond to additional IGF-I with a proliferation response, indicating a shutdown in the IGF-I pathway that warrants further investigation. For collagen I, it has been shown that a gel will induce changes in the secretion of IGFBPs, modulators in the IGF-I signaling pathway. Further investigation will reveal if these secretory changes are due to the shape and positioning of the cells or due to the presentation of the collagen I. These studies have implications for researching and developing possible therapeutic modalities to modify cell response with the end result of increased growth (for agricultural purposes) or decreased growth (to slow cancerous proliferation). Possible future therapeutics to modify IGF-I pathway components might range from pharmacological interventions, both local and systemic, to the introduction of a scaffolding or tethering of molecules at a treatment site.

6.3 References

1. Farrelly, N., Lee, Y., Oliver, J., Dive, C., and Streuli, C.H. (1999). Extracellular matrix regulates apoptosis in mammary epithelium through a control on insulin signaling. *J. Cell Biol.* **144**, 1337-1347.
2. Perrone, C.E., Fenwick-Smith, D., and Vandenburgh, H.H. (1995). Collagen and stretch modulate autocrine secretion of insulin-like growth factor-1 and insulin-like growth factor binding proteins from differentiated skeletal muscles. *J. Biol. Chem.* **270**, 2099-2106.
3. Chen, C. S., Mrksich, M., Huang, S., Whitesides, G. M., and Ingber, D.E. (1997). Geometric control of cell life and death. *Science.* **276**, 1425-1428.

APPENDIX A

Calculation of binding parameters R, K_D, and K_N from IGF-I / IGF-IR binding studies (Chapters 2 and 3)

Binding studies were conducted to determine the total number of available IGF-I receptors (R) present on the surfaces of mammary epithelial cells and to calculate the dissociation constant (K_D) of IGF-I to the receptor. To prevent internalization of receptors, cells were cooled to 4 C. This low temperature slows all cellular processes; receptor internalization, degradation, synthesis, and intracellular trafficking become negligible. A simple steady state model with IGF-I binding reversibly to the IGF-IR was assumed *(1)*. Nonspecific binding was assumed to be reversible and non-saturable.

Cells were prepared by plating at known seeding densities to 24-well plates and by allowing adherence to the plates over the next 24 h in media with 10% fetal bovine serum. After 24 h, media was replaced with serum–free media to "starve" the receptors for 72 h. After cooling plates to 4 C, cold IGF-I was added in concentrations of 0, 2, 4, 8, 16, 32, 16, and 215 ng/well to different wells. To each well was added 2 ng of the radioactively labeled IGF-I, [^{125}I] IGF-I. Plates were incubated at 4 C for 3 h to achieve steady state binding. After the incubation period, cells were washed, lysed, and counted for remaining radioactivity by gamma counter.

Two rate equations were assumed. One equation described the binding of IGF-I with the IGF-IR (EQ. 1) and the other equation described the nonspecific binding to the surface (EQ 2).

$dC/dt = k_{forward}LR - k_{reverse}C$ (EQ. 1)

$dB/dt = k_{forwardnon}L - k_{reversenon}B$ (EQ. 2)

where

C = complexed receptor and ligand
L = free ligand available for binding
R = unbound or free receptors
R_T = total receptors
$k_{forward}$ = association rate constant
$k_{reverse}$ = dissociation rate constant
B = complexed ligand and nonspecific surface site
$k_{forwardnon}$ = nonspecific association rate constant
$k_{reversenon}$ = nonspecific dissociation rate constant

Equilibrium steady state was assumed for EQ. 1 and EQ. 2, and the total bound IGF-I was assumed equal to specific bound IGF-I plus non-specific bound IGF-I. Also assumed was that ligand depletion was negligible, allowing L to be replaced by L_o. It was assumed that no degradation or synthesis of receptors occurred, so that R could be replaced with R_T-C. Thus, $dC/dt = k_{forward}L(R_T-C) - k_{reverse}C$. Total bound complexed ligand could then be calculated:

$T = C + B = [(R_T L_o)] / [K_D + L_o] + [K_N] L_o$ (EQ. 3)

where

T = total bound complexed ligand
L_o = total available ligand
$K_D = k_{reverse} / k_{forward}$
$K_N = k_{forwardnon} / k_{reversenon}$

EQ. 3 was solved for R, K_D, and K_N using a least-squares fit of the experimental data with a nonlinear regression in Mathematica® (Wolfram, Champaign, IL.) The resultant

estimated values for R_T, K_D, and K_N yielded calculated values for L_o that closely approximated the experimental values and gave confidence to the model

The calculation was entered into Mathematica® as follows:

```
<<Statistics`NonlinearFit`
NonLinearRegress[data, ((R*(x+2)/(KD+x+2) +
KN*(x+2))/(x/2+1)),{x},{{KN,0,1000},{KD,0,1000},(R,0,1000}}]
```

In the command line, “data” was the data set for added cold IGF-I and corresponding radioactive count for each well set up as a two dimensional matrix of data. The value for available ligand L_o was equal to (x + 2) where x was the amount of cold IGF-I added to each well and “2” is added for the 2 ng of radioactively labeled [^{125}I] IGF-I, which was added to every well. K_N, K_D, and R were all specified to have values in the range of 0 to 1000 to facilitate the calculation for meaningful estimates for these parameters. The advantage of using this experimental approach and calculation to calculate K_N, K_D, and R is that the entire experimental data set was used for a better model fit to the experimental data.

References

1. Lauffenburger, D.A. and Linderman, J.J. (1993). Receptors: models for binding, trafficking and signaling. Oxford University Press, New York.

APPENDIX B

Procedure for detection of IGFBP proteins by ligand blot

The goal of the ligand blot procedure is to detect insulin-like growth factor binding proteins (IGFBPs) in cell conditioned media. The exposure of growth media to living cells is referred to as "cell conditioning" of the media. In our studies, MAC-T mammary epithelial cells were grown in culture, growth media was exposed to these cells for set lengths of time, and the media was collected and processed for analysis using a ligand blot procedure. The following protocol was developed in Dr. R. M. Akers' laboratory at Virginia Tech.

For our studies, MAC-T cells were seeded on three different substrates in 24-well plates. These substrates included tissue culture treated plastic surfaces, Becton Dickinson collagen I (rat-tail) pre-coated surfaces, or collagen I (rat-tail) 300 uL gels prepared from an in-house rat-tail collagen I preparation *(1)*. Cells were seeded at a density of 50,000 or 200,000 cells per well in Dulbecco's modified Eagle's media with 10% fetal bovine serum (DMEM + 10% FBS, Gibco, USA). The cells were allowed to adhere for 24 hrs after seeding, and the media was then aspirated and discarded. Media was replaced with 1 mL plain DMEM (i.e. no FBS) per well. For the next 48 hrs, the MAC-T cells conditioned the media. After the 48 hr conditioning period, the media was collected and centrifuged at 1000x g for 10 min to remove cell debris and proteases. The supernatant,

with the cell-secreted IGFBPs, was collected in microcentrifuge tubes and was stored frozen at -20 C.

Frozen supernatant was thawed and lyophilized overnight in a Savant Speed-Vac® concentrator. The lyophilized pellets (each resulting from 1 mL conditioned media) were each reconstituted with 50 μL of de-ionized water. To prepare samples for the ligand blot, 20 μL of each reconstituted solution was added to 20 μL of an SDS buffer solution with bromophenol blue dye (recipe below). These samples were heated in a 60 C water bath for 10 min prior to running on an SDS polyacrylamide (PAGE) gel (12.5%) to denature the proteins and facilitate the molecular weight based separation on the gel.

Detailed steps for the ligand blot follow. The resulting blots were scanned on an Epson Expression 800 scanner and analyzed using the software program GelWorks 1D Intermediate version 4.01 (Ultra Violet Products, NonLinear Dynamics, Ltd.) to measure the intensities of the resultant IGFBP bands on the ligand blots. By measuring these band intensities using the densitometry software, relative amounts of MAC-T secreted IGFBPs were determined and compared for the different initial cell seeding densities and cell seeding surfaces as described in Chapter 6.

Part 1: Polyacrylamide gel (PAGE) electrophoresis

Gel preparation

Gels were prepared for 1-D electrophoresis on the SE 600 Standard Vertical Unit, Hoefer Scientific Instruments (USA). To form the lower polyacrylamide gel (12.5% resolving gel) and upper polyacrylamide gel (3.9% stacking gel) the following solutions were prepared:

<u>Solution "A"</u>

100 mL de-ionized H_2O

1.6 g BIS acrylamide (N,N'-Methylene-bis-acrylamide, electrophoresis purity reagent, Bio-Rad, USA)

60 g acrylamide (electrophoresis reagent, ≥ 99%, Sigma, USA)

Stirred to dissolve; additional de-ionized water added to bring volume to 200 mL. Filter sterilized. Stored at 4 C, protected from light with aluminum foil.

<u>Solution "B"</u>

50 mL de-ionized H_2O

18.17 g Trizma Base® (reagent grade, Sigma, USA)

Stirred to dissolve; additional de-ionized water added to bring volume to 100 mL. pH'd to 8.8 with HCl. Filter sterilized. Stored at 4 C.

Solution "C"

50 mL de-ionized H_2O

6.06 g Trizma Base® (reagent grade, Sigma, USA)

Stirred to dissolve; additional de-ionized water added to bring volume to 100 mL. pH'd to 6.8 with HCl. Filter sterilized. Stored at 4 C.

10% SDS solution

150 mL de-ionized H_2O

20 g lauryl sulfate (sodium dodecyl sulfate, approx 99%, Sigma, USA)

Stirred to dissolve; additional de-ionized water added to bring volume to 200 mL. Stored at room temperature.

10% Ammonium persulfate (prepared just prior to use)

0.1 g ammonium persulfate (Bio-Rad, USA)

1 mL de-ionized H_2O

H_2O saturated butanol

Added n-butanol (Sigma, USA) to de-ionized H_2O in a 1:2 ratio in a 100 ml bottle and agitated well. The water saturated butanol layer was in the upper layer and was aspirated by syringe as needed in forming the gel.

The 12.5 % polyacrylamide resolving gel was prepared in a 125 mL side-arm flask. For the preparation of two gels: 19.6 mL of de-ionized H_2O, 25 mL solution "A", and 14.7 mL solution "B" were added to the flask. The flask was placed on a stir plate with suction to de-gas for 10 min. The contents were then divided to two small beakers. To each beaker, 150 uL 10% SDS solution and 200 uL 10% ammonium persulfate were added. Just before pouring, 13 ul Temed (N,N,N',N'-Tetramethylethylenediamine, electrophoresis reagent, Sigma, USA) was added to one beaker. The solution was drawn up in a 50 cc syringe with an attached needle. Solution was ejected carefully between the glass plates for the first gel and was immediately topped with a small amount of H_2O saturated butanol from a second syringe. The saturated butanol removed surface bubbles for a clean edge on the resolving gel. The procedure was repeated for the second gel, and the gels were allowed to polymerize for at least one hr.

H_2O saturated butanol was drained from the tops of the gels by inverting the apparatus. A 15-well comb was positioned between the glass plates of each gel to form the sample wells in the stacking gels.

The 3.9 % polyacrylamide stacking gel was prepared similarly to the 12.5 % resolving gel. To a 125 mL side-arm flask, 12.4 mL of de-ionized H_2O, 2.6 mL solution "A", and 4.9 mL solution "C" were added to the flask. The flask was placed on a stir plate with suction to de-gas for 10 min. The contents were divided into two small beakers. To each beaker, 50 μL 10% SDS solution and 300 μL 10% ammonium persulfate were added. Just before pouring, 10 μL Temed was added to one beaker. The solution was drawn up

in a 20 cc syringe with an attached needle. The solution was ejected carefully between the glass plates above the 12.5% resolving gel. The procedure was repeated for the second gel, and the gels were allowed to polymerize for at least one hr.

Sample preparation

To prepare samples for the ligand blot, 20 μL of each reconstituted solution was added to a separate microcentrifuge tube; 20 μL of an SDS buffer solution with bromophenol blue dye (recipe below) was added to each of these microcentrifuge tubes. These samples were heated in a 60 C water bath for 10 min prior to loading on the SDS polyacrylamide (PAGE) gel.

SDS buffer with bromophenol blue dye

2.5 mL 0.5 M Tris (pH 6.8)*

4.0 mL 10% SDS solution

2.0 mL glycerol (glycerin, 99%, Sigma, USA)

0.1 mg bromophenol blue (Fisher Scientific, USA)

Stirred to dissolve; additional de-ionized water was added to bring the volume to 10 mL.

Stored at room temperature.

* 0.5 M Tris stock: 6.055g Trizma Base® (reagent grade, Sigma, USA) was added to 75 mL de-ionized H_2O. The solution was pH'd to 6.8 with HCl, and additional de-ionized

water was added to bring the volume to 100 mL. The solution was stored at room temperature.

Electrophoresis

<u>Running buffer solution</u>

For each liter of running buffer, 2.8 g Trizma Base® (reagent grade, Sigma, USA), 14.3 g glycine (99%, TLC, Sigma, USA), 2.0 g lauryl sulfate (sodium dodecyl sulfate, approx 99%, Sigma, USA) were added to 800 mL de-ionized water on stir plate. Additional de-ionized water was added to bring the volume to 1 L. Stored at room temperature.

The electrophoresis set-up (SE 600 Standard Vertical Unit, Hoefer Scientific Instruments, USA) was run for 18 hr at 60 V.

Part 2: Transfer to nitrocellulose membrane

<u>Transfer buffer solution</u>

For each liter of transfer buffer, 3.0 g Trizma Base® (reagent grade, Sigma, USA), 14.4 g glycine (99%, TLC, Sigma, USA), 200 mL methanol (biotech grade, FisherBiotech, USA) was added to 750 mL de-ionized water and mixed on a stir plate. Additional de-ionized water was added to bring the volume to 1 L. The solution (pH 8.3) was stored at room temperature.

Transfer

Proteins were transferred from the gel to a nitrocellulose transfer membrane (NitroPure, Osmonics, Inc., Westborough, MA) on a Trans-Blot transfer cell (Bio-Rad, USA) for 4 hr at 50V.

Part 3: Post transfer washes and probe with [^{125}I]IGF-I

The nitrocellulose transfer membrane was removed from the transfer apparatus and blotted between two pieces of filter paper for 10 min at 4 C.

Post transfer wash solutions

Saline stock solution (5X)

60.5 g Trizma Base® (reagent grade, Sigma, USA)

45.0 g sodium chloride (FisherChemical, USA)

2.5 g sodium azide (99.5%, Sigma, USA)

500 mL de-ionized H_2O

Stirred on stir plate. Additional de-ionized water was added to bring the volume to 1 L. (pH 7.4)

Wash “1”

100 mL saline stock solution (5X) and 15 mL IGEPAL CA-630 (Sigma, USA) were added to 385 mL de-ionized H_2O and stirred on a stir plate. Additional de-ionized water was added to bring the volume to 500mL.

Wash “2”

100 mL saline stock solution (5X) and 5 g bovine serum albumin (Sigma, USA) were added to 300 mL de-ionized H_2O and stirred on a stir plate. Additional de-ionized water was added to bring the volume to 500 mL.

Wash “3”

100 mL saline stock solution (5X) and 0.5 mL Tween 20 (polyoxyethylenesorbitan monolaurate, Sigma, USA) were added to 300 mL de-ionized H_2O and stirred on a stir plate. Additional de-ionized water was added to bring the volume to 500 mL.

Post transfer washes

All containers of washes were placed in a 4 C cold room, and membranes were placed in each wash sequentially on a platform rocker for the designated time: Wash “1” for 30 min, then Wash “2” for 2 hr, and finally Wash “3” for 10 min.

[^{125}I]IGF-I probe

12 mL of the saline stock solution (5X), 0.6 g bovine serum albumin, and 0.06 mL Tween 20 were added to sufficient de-ionized water to bring the volume to 60 mL. The [^{125}I]IGF-I probe solution was prepared by adding sufficient iodinated IGF-I to the buffer solution to achieve a solution radioactivity count of 900,000 cpm/mL. The membranes were incubated in the [^{125}I]IGF-I probe solution on a platform rocker at 4 C overnight (12 to 16 hrs).

Post [^{125}I]IGF-I probe washes

The membranes were washed in 100 mL Wash "3" solution (recipe above) for 15 min at room temperature on a platform rocker. Used wash solution was discarded to radioactive waste, and the wash repeated with new Wash "3" solution.

A 1X saline solution was prepared by adding 100 mL saline stock solution (5X) to 400 mL de-ionized water. This solution was used to wash the membranes for 15 min at room temperature on a platform rocker; used wash was discarded to radioactive waste each time. The wash was repeated 2 more times with 1X saline solution.

Dry blot

Membranes were placed between two pieces of filter paper in a 37 C oven for 2 hrs to dry.

Part 4: Exposure of film for ligand blot

Membranes were wrapped in plastic wrap and placed in cassettes with X-OMAT™ AR scientific imaging film (Eastman Kodak Company, NY) to expose films at -70 C for 3 to 5 days. The film was developed using standard film development techniques, and the scanned image of the developed film was saved as a .tif file for analysis using densitometry software.

References

1. Richards J., Larson L., Yang, J., Guzman, R., Tomooka, Y., Osborn, R., Imagawa, W., and Nandi, s. (1983). Method for culturing mammary epithelial cells in a rat tail collagen gel matrix. *J. Tissue Cult. Methods* 8, 31-36.

APPENDIX C

Procedure for mRNA detection by Northern blot

Northern blots were performed to detect expression of IGFBP-2 and IGFBP-3 mRNA by MAC-T mammary epithelial cells seeded at 200,000 cells/well in 24-well plates with 3 different cell seeding surfaces: tissue culture treated plastic plates, Becton Dickinson collagen I (rat-tail) pre-coated plates, or collagen I (rat-tail) 300 μL gels prepared in-house. Cells were seeded in Dulbecco's modified Eagle's media with 10% fetal bovine serum (DMEM + 10% FBS, Gibco, USA) and allowed to adhere. After 24 hrs, the media was removed, the cells were washed once with 1 mL PBS, and plain DMEM (1 mL/well) was added. After an additional 24 hrs, either 100 μL plain DMEM with or without 100 ng IGF-I was added to the wells, and plates were incubated in a humidified incubator for another 24 hrs at 37 C and 5% CO_2.

To harvest the RNA from the cells, the media was removed, and 200 μL of TRI REAGENT® (Molecular Research Center, Inc., Cincinnati, OH) was added to each well. The TRI REAGENT® lyses the cells, and the lysates were passed several times through a pipette tip to help break up the material. Lysates were then removed to microcentrifuge tubes by pipette. The contents of 3 or 4 wells were pooled for each treatment.

Part 1: RNA isolation procedure

The following procedure was followed for RNA isolation as recommended by the manufacturer of TRIZOL® reagent (GIBCO BRL Life Technologies), a reagent similar to TRI REAGENT®:

160 µL chloroform was added to each microcentrifuge tube containing 800 µL cell lysate in TRI REAGENT®. The tubes were capped and shaken vigorously by hand for 15 sec and incubated at room temperature for 2 to 3 min. The tubes were then centrifuged at 12,000x g for 15 min at 4 C. (The RNA separates to the upper, or aqueous, phase in this procedure.) The aqueous phase in each tube was transferred to a new microcentrifuge tube, and 400 µL of 70% isopropanol was added to each tube to precipitate the RNA. The tubes were then incubated at room temperature for 10 min, followed by centrifugation at 12,000x g for 10 min at 4 C. A very small, soft pellet was visible at the bottom of each microcentrifuge tube after this step.

The supernatant was removed by carefully inverting each tube to let the liquid drain out. The pellets were washed by adding 1 mL of 75% ethanol to each tube, vortexing each tube briefly to dislodge the pellet, and centrifuging the tubes at 7500x g at room temperature for 5 min. The supernatant was removed by carefully allowing the liquid to drain from the tubes without dislodging the pellets. Tubes were left to air-dry 5 to10 min before proceeding. The RNA pellets were re-dissolved by adding 50 µL of RNAsecure™

Resuspension Solution (Ambion, Inc., Austin, TX) to each microcentrifuge tube and incubating the tubes in a 60 C water bath for 10 min. Capped tubes were stored at -70 C.

Part 2: Determination of RNA concentration for gel loading

The microcentrifuge tubes of pellets reconstituted with RNAsecure™ Resuspension Solution were placed on ice. 1 μL of each reconstituted pellet was added to 99 μL of TE buffer in a new microcentrifuge tube. The optical density was read for each of these samples on a spectrophotometer at wavelengths 260 and 280. (The spectrophotometer was autozeroed with 100 μL of TE buffer.) Ratios of the optical densities (wavelength 260/wavelength 280) were calculated for each tube. Ratios of RNA/DNA greater than 1.8 are desirable; otherwise suggests that the sample contains nucleic acids, proteins, or contaminants (REF: Basic Methods in Molecular Biology 2nd edition, Leonard G. Davis, W. Michael Kuehl, James F. Battey, APPLETON & LANGE, Norwalk, CT 1994).

Total RNA loaded per lane on the gel was at least 15 μg/mL. To calculate μg/μL RNA in each sample, the following formula was used:

(optical density at wavelength 260) × (100 μL 1 μL) × (40 μg RNA 1 mL) × (1 mL 1000 μL) = μg/μL RNA in the sample

where:

optical density at wavelength 260: this reading was taken from the spectrophotometer for 1 μL of the reconstituted pellet in 99 μL of DEPC water

100 μL 1 μL: the ratio of 1 μL of the reconstituted pellet + 99 μL of DEPC water divided by 1 μL of the reconstituted pellet

40 μg RNA 1 mL: the concentration of RNA at wavelength 260 with optical density = 1.0

1 mL 1000 μL: a conversion factor

From the resultant calculated concentration, the required volume of solution from each reconstituted pellet was determined. These samples were lyophilized and reconstituted in the microcentrifuge tubes as follows:

5 μL water

15 μL Formaldehyde Load Dye (Ambion, Inc., Austin, TX)

0.5 μL ethidium bromide (EtBr)

The microcentrifuge tubes were heated in a 65˚C water bath for 10-15 min. Then, the microcentrifuge tubes were placed on ice, and the contents were loaded immediately onto the gel.

Part 3: Electrophoresis of RNA samples

The gel was run at 100 V for 2 hrs. 28S and 18S bands stained with EtBr were visualized by photographing the gel under UV light.

Running buffer solution

100 mL 10x MOPS

50 mL formaldehyde

850 mL DEP treated water

MOPS solution

0.1 M MOPS

80 mM sodium acetate

10 mM EDTA

MOPS solution was prepared by first adding MOPS to 100mM sodium acetate. Sodium hydroxide was used to pH the solution to 7.0. Next DEP treated 0.5 M EDTA at pH 8.0 was added. Water was added to bring solution to desired volume.

1% agarose gel

2 g agarose (for molecular biology, Sigma, USA) was melted in 144.2 mL DEP water in a flask and placed in a 60˚C water bath to reduce the temperature. Under the fume hood, 20 mL 10x MOPS and 36 mL 37% formaldehyde were added to the flask. The contents of the flask were swirled carefully to avoid bubbles while mixing and then poured into a gel former.

Part 4: RNA transfer to membrane

The gel was washed in 500 mL of 10x SSC solution for 30 min to remove the formaldehyde.

Transfer buffer (20x stock)

3 M sodium chloride

0.2 M sodium citrate

The transfer set-up was made by placing the gel former upside-down in a large glass baking dish. Two sheets of filter paper cut the width of the gel former and twice the length of the gel former were place over the gel former, so that the paper hung over the ends into the baking dish. 500 mL 10x SSC solution was added to the dish. The gel was placed with the RNA side up onto the filter paper. Pre-wetted Hybond N^+ membrane (Amersham Pharmacia Biotech, England) was placed on top of the gel, and a sheet of

plastic wrap was used to cover the whole plate. A square was cut in the center of the plastic wrap to allow the Hybond N^+ membrane to be exposed. The cut was made just inside of the perimeter of the membrane to prevent solution from wicking up the sides of the membrane. Four layers of filter paper cut to the size of the membrane were placed on top of the membrane. This was followed with a layer of paper towels (about 1.5 in thick), a piece of Plexiglas, and a glass bottle of water for a weight. Transfer occurred overnight (12 to 16 hrs).

The membrane was cross-linked in a UV cross-linker and then washed in 2x SCC buffer for 5 min. Liquid was drained from the membrane, and the membrane was stored at -20˚C until ready to probe.

Part 5: Hybridization of the membranes and exposure of film for the Northern blot

Preparation of mRNA probes

Probes were made using the Promega Prime-a-Gene® Labeling System (Promega, Madison, WI) using probe inserts for IGFBP-2, IGFBP-3, and the GAPDH housekeeping gene at known concentrations.

Hybridization of membrane

The membranes were pre-hybridized by placing the membranes in roller tubes with the RNA side of the membrane toward the center. 20 mL of Quickhyb® (Stratagene®, Cedar Creek, TX) was added to each tube to wet the membrane surface. The tubes were placed in a hybridization oven with rollers (Rock'n'Roll Hybridization oven, Boeckel Scientific) for 20 min at 68˚C. After the membrane pre-hybridization, the roller tubes were taken out and half of the probe mixture was added to each tube. The tubes were returned to the hybridization oven and incubated for 1 hr at 68˚C. The membranes were then washed twice for 25 min per wash with 100 mL 2x SSC + 0.1% SDS solution in the hybridization oven with the temperature set at room temperature (21˚C). This was followed with a 30 min wash at 60˚C with 100 mL 0.1x SSC + 0.1% SDS solution.

The membranes were removed from the roller tubes, wrapped in Saran Wrap, and placed in cassettes with Kodak X-OMAT film overnight at -80˚C. The film was developed using standard film development techniques. The scanned image of the developed film was saved as a .tif file for analysis using densitometry software.

Stripping the membrane to re-probe

The membrane can be stripped of an RNA probe so that it can be analyzed with another RNA probe. To do this, a flask of 300 mL of 0.1 x SSC + 0.1% SDS solution was heated in a microwave until the solution was boiling. The membranes were placed in a glass

pyrex baking dish and washed with the hot buffer for 15 min. This was repeated with fresh, hot buffer. The membrane was then stored at -20˚C until probing with the next RNA probe.

APPENDIX D

Procedure for detection of IGF-IR by Western blot

Detection of insulin-like growth factor-I receptors (IGF-IR) in MAC-T mammary epithelial cell lysates was performed using a Western blot procedure. The following protocol was developed in Dr. W. R. Huckle's laboratory at Virginia Tech.

Part 1: Preparation of cell lysates

The cell lysates were prepared from MAC-T cells grown in 6-well plates. For our studies, MAC-T cells were seeded on three different substrates. These substrates included tissue culture treated plastic surfaces, Becton Dickinson collagen I (rat-tail) pre-coated surfaces, or collagen I (rat-tail) gels prepared from an in-house rat-tail collagen I preparation *(1)*. The cells were seeded at a density of 250,000 cells/well in 3 mL/well Dulbecco's modified Eagle's media with 10% fetal bovine serum (DMEM + 10% FBS). Cells were grown to confluency over the next 2 or 3 days. Media was changed with 3 mL/well plain DMEM (i.e. no FBS) to initiate serum starvation. After 48 hrs, the IGF-I treatments were performed. For a phosphorylated protein control, vanadate (20μM) and peroxide (1 mM) were added to a control well of cells.

After the IGF-I exposure time period, the media was dumped out of the plates by inverting the plates over a waste container. No wash step was performed, and the plates

were placed on ice. Any remaining liquid in the well was quickly aspirated by pipette. The phosphorylation was quenched by adding 0.5 mL of ice-cold lysis buffer to each well. A cell scraper was used to remove the cells from the bottom surface of each well. Then the slurry of cell lysates from each well was aspirated to autoclaved microcentrifuge tubes. These tubes were centrifuged at 14,000x g for 15 min to remove particles. The supernatant was removed and frozen at -70 C until ready for the next step.

Lysis buffer

20 nM HEPES

10 mM sodium fluoride

1 mM sodium vanadate

10% glycerol

500 mM sodium chloride

5 mM EDTA

1% Triton X-100

1% Protease Inhibitor Cocktail (Sigma-Aldrich Product, USA)

All chemicals were combined and pH'd to 7.5. Buffer was stored at 4 C.

Part 2: Immunoprecipitation of cell lysates

Each mL of cell lysate was incubated with 10 μL of primary antibody (IGF-IRβ (C20), Santa Cruz, cat # SC 713) in a microcentrifuge tube on a rocker platform for 1 hr at 4 C

to precipitate the bovine IGF-IRβ. 20 μL of well-mixed Protein A-agarose suspension was added to each microcentrifuge tube. The tubes were capped and returned to the rocker platform at 4 C for an additional 1 hr incubation. Tubes were then centrifuged at 500x g (2500 rpm on a Beckman microfuge) for 5 min at 4 C. The supernatant was discarded from each microcentrifuge tube using a tuberculin syringe, using care so that the pellet was not dislodged.

The pellet of each microcentrifuge tube was washed with a small amount (0.2 to 0.5 mL per pellet) lysis buffer prepared without the protease inhibitors. Tubes were centrifuged and the supernatant discarded, again using a tuberculin syringe so that the pellet was not dislodged. The wash step was repeated. Tubes were placed in a heat block for 2 to 3 min at 100 C to denature the proteins. Samples were stored frozen at –20 C. Before loading onto a gel, the samples were thawed and heated again on a heat block for 2 to 3 min at 100 C.

Part 3: Polyacrylamide gel (PAGE) electrophoresis

Samples were run on a BioRad mini-cell set using pre-cast 10-well 10% TR-GL gels. 20 to 30 μL of sample were loaded in each of the 10 wells. The unit was run at 125 V for 1.25 hrs.

Running buffer solution

25 mM Tris

192 mM glycine

0.1% (w/v) SDS

pH 8.3

Part 4: Transfer to PVDF membrane

After the electrophoresis, the gels were removed and placed in a BioRad mini-cell set transfer apparatus with a polyvinylidene difluoride membrane (PVDF membrane) (Millipore, Bedford, MA). The transfer unit was run at 100 V for 1 hr.

Transfer buffer solution

25 mM Tris

192 mM glycine

20% (v/v) methanol

pH 8.3

Part 5: Probing with antibodies

Overnight blocking of membrane

Membranes were placed in a bath of 50 mL of blocking solution on a platform rocker overnight (16 hrs) at 4 C.

Blocking solution

20 mM Tris

150 mM sodium chloride

0.05% Tween 20

0.2% gelatin

Chemicals were combined and pH'd to 7.5.

Addition of primary antibody

Primary antibody (10 μL IGF-IRβ (C20) from Santa Cruz cat # SC 713 **OR** 5 μL P-TYR-100 from NEB products) was added to 10 mL of the blocking solution used for the overnight blocking to make the primary antibody solution. The membrane was incubated in the primary antibody solution on a platform rocker for 1 hr at room temperature. Then the membrane was washed in 50 mL of wash solution on a platform rocker at room temperature for 10 to 15 min. This was repeated 3 more times with fresh wash solutions.

Wash solution

20 m*M* Tris

150 m*M* sodium chloride

0.05% Tween 20

Chemicals were combined and pH'd to 7.5.

Addition of secondary antibody

Secondary antibody (0.5 μL) was added to 10 mL of the blocking solution used for the overnight blocking to make the secondary antibody solution. The membrane was incubated in the solution on a platform rocker for 1 hr at room temperature. Then the membrane was washed in 50 mL of wash solution on a platform rocker for 10 to 15 min at room temperature. This was repeated 3 more times with fresh wash solutions.

Part 6: Addition of horseradish peroxidase (HRP) conjugate and exposure of films

The membrane was removed with a set of tongs from the wash solution, and the fluid was allowed to drain from the membrane to a paper towel. This step removed excess moisture prior to addition of the horseradish peroxidase (HRP) conjugate. The membrane was placed on a flat surface, and 8 mL/membrane of the prepared working

solution of SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) was added to completely wet the membrane as recommended by the manufacturer. The membrane incubated in the solution for 5 min at room temperature, and then the membrane was lifted by tongs to drain the excess liquid to a paper towel. The membrane was wrapped in plastic wrap and placed in an exposure cassette with Kodak BioMax™ film (Eastman Kodak Co., Rochester, NY). The film was developed using standard techniques within a few minutes of exposure time. Ten to fifteen minutes of film exposure usually gave optimal signals.

References

1. Richards J., Larson L., Yang, J., Guzman, R., Tomooka, Y., Osborn, R., Imagawa, W., and Nandi, s. (1983). Method for culturing mammary epithelial cells in a rat tail collagen gel matrix. *J. Tissue Cult. Methods* 8, 31-36.

APPENDIX E

Radioimmunoassay (RIA) procedure for IGF-I detection

An IGF-I radioimmunoassay technique was used to determine if IGF-I was secreted by MAC-T mammary epithelial cells into conditioned media. The samples used for detection of IGFBP-2 and IGFBP-3 by ligand blot were re-lyophilized and then reconstituted with 100 μL of de-ionized water for the RIA assay.

Part 1: Extraction for IGF-I

Each 100 μL sample was vortexed, and 900 μL of extraction mixture was added to each microcentrifuge tube. This was vortexed and incubated at room temperature for 1 hr. Next, the microcentrifuge tubes were centrifuged at 13,290x g (12,000 rpm on a Beckman microfuge) for 10 min at 4 C. 500 μL from each microcentrifuge tube was transferred to a glass 12x75 mm tube. 200 μL of 0.855 M Tris Base was added to each glass tube to neutralize the samples. The samples were then incubated in a -20 C freezer for 1 hr. After the 1 hr incubation, the glass tubes were immediately centrifuged at 5500x g (4,000 rpm in a Beckman J6-B clinical centrifuge) for 30 min at 4 C. The supernatant of each glass tube was decanted into a plastic 12x75 mm tube. These decanted supernatants were the extracted samples. Extracted samples were stored at -20 C.

Part 2: IGF-I RIA Assay

Extracted samples were diluted with buffer by first adding 470μL of IGF-I RIA buffer to glass 12x75 mm tubes. For each sample, 30μL of well-mixed extracted sample was added to the corresponding glass tube. Tubes were numbered from # 42 to the end.

Standard curve

IGF-I concentrations in the samples were determined using a standard curve with each assay. The standard was prepared using IGF-I. 41 glass 12x75 mm tubes were numbered from 1 to 41.

IGF-I RIA buffer was added to tubes 6 to 41 in the following amounts:

Tubes #	Volume (μL)
6-9	500
10-13	497.5
14-17	495
18-21	490
22-25	480
26-29	460
30-33	420
34-37	340

38-41	320

The IGF-I standard was added in amounts to bring the combined volume to 500 μL:

Tubes #	Volume (μL)
6-9	0
10-13	2.5
14-17	5
18-21	10
22-25	20
26-29	40
30-33	80
34-37	160
38-41	320

Addition of antibodies and analysis

100 μL mouse control serum was added as a control to tubes #3, 4, and 5. To tubes #6 to the end, 100 μL of the primary antibody) was added. The rack of tubes was shaken carefully by hand to mix the contents. Next, 100 μL of [^{125}I]IGF-I (approximately 250 counts/ μL) was added to each tube, numbered from 1 to 41. Again, the rack of tubes was shaken carefully by hand to mix contents. The tubes were covered with aluminum foil, and incubated for 24 hrs at 4˚C.

After 24 hrs, 100 μL of the secondary antibody (goat anti-mouse 1/20) was added to tubes # 3 to the end. The rack of tubes was shaken to mix and returned to 4˚C to incubate for 72 hrs. Next, 1 mL of DD-PBS was added to tubes # 3 to the end. All tubes were centrifuged except # 1 and 2 (which are the total count controls) at 1850 x g (3000 rpm in a Beckman J6-B clinical centrifuge) for 30 min at 4 C. After centrifuging, the tubes were placed back in the rack. Using a wire cover over the rack, the rack was inverted over a radioactive waste container to drain the supernatant. While still inverted, the tube ends were washed in a water rinse, and the rack was placed upside down on absorbent paper to dry. When dry, the tubes were loaded onto a gamma counter, and the radioactivity of [^{125}I]IGF-I in each tube was counted. A standard curve for IGF-I content was created from tubes #1 through 41. IGF-I content in each extracted sample was calculated and recorded in ng/mL.

APPENDIX F

Statistical analyses of results

DNA Analysis

All samples were measured in duplicate and the duplicates were averaged. These averages were then averaged and standard error of the mean of the averages was calculated using Microsoft® Excel software.

Tests for Significance in Treatment Comparisons

Statistical analyses for significance ($p<0.05$) were performed with a Bonferroni (Dunn) T-test and specific contrasts using SAS software (SAS Institute).

VITA

of

Rose Marie Robinson

Rose Marie Robinson graduated from the University of Maryland in College Park with a Bachelor of Science in Chemical Engineering in June 1987 under a co-op program with the Naval Surface Warfare Center in Dahlgren, VA. Following graduation, she worked as a chemical engineer at the Naval Surface Warfare Center for two years.

In 1989, Ms. Robinson re-located to Southwest Virginia and entered nursing school at Virginia Western Community College in Roanoke, VA. She obtained an Associate Degree in Nursing and became a registered nurse in 1991 while working as a nursing assistant on a medical surgical floor. From June 1991 to July 1996, she worked in the cardiac care unit at Carilion Roanoke Memorial Hospital, becoming certified as a critical care registered nurse and as an advanced cardiac life support instructor.

In August 1996, Ms. Robinson began full-time studies as a graduate student in the Department of Chemical Engineering at Virginia Polytechnic and State University in Blacksburg, VA and earned a Master of Science in Chemical Engineering in December 1998. From August 2002 to May 2004, she worked as an adjunct faculty member in the Division of Engineering Fundamentals at Virginia Tech.