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Crabp1 Regulation of FAS RNA in Mouse Mammary Gland ASCs

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Abstract:

Introduction: The cellular retinoic acid binding protein 1 (Crabp1) promotes tumor growth and is expressed in adipocyte stromal cells (ASCs-L) present during lactation. One known function of Crabp1 is the binding and retention of retinoic acid (RA) in the cytoplasm. This can prevent RA regulated transcription of other genes. Research has shown that Crabp1 is also responsible for the inability of ASC-Ls to retain lipid. This study hoped to connect these two functions of Crabp1 and hypothesized that Crabp1 limits lipid accumulation through Fatty acid synthase (*FAS*) transcriptional regulation. *FAS* is an example of a gene that is regulated by RA.

Methods: Luciferase assays, RT-qPCR, and Oil Red O assays were performed to study *FAS* promoter activity, mRNA levels, and lipid accumulation, respectively. ASCs at different stages of mammary gland development were studied to determine the relationship between Crabp1, *FAS*, and lipid accumulation upon RA treatment.

Results: Our results suggest that RA treatment leads to a decrease in *FAS* promoter activity as well as *FAS* and *Crabp1* mRNA levels in ASC-Nulliparous. Furthermore, higher *Crabp1* mRNA levels in ASC-Ls did not correlate with a decrease in *FAS* mRNA. ASC-Ls also showed low lipid accumulation in the presence of adipocyte differentiation media, suggesting that Crabp1 regulates lipid accumulation, but not through the regulation of *FAS.*

Conclusion: The mechanisms of Crabp1 in lipid regulation of ASCs remains to be determined. Our data show that Crabp1 does not regulate *FAS* though RA retention in ASC-Ls. However, our results are consistent with the literature in that ASC-Ls have high levels of Crabp1, but low lipid accumulation. Through understanding how Crabp1 is acting on the adipocytes in the mammary gland it can be better understood what its tumor promoting function is in PABCs.

Introduction:

Cancer is a broad term used to describe diseases in which cells grow out of control, destroying body tissue. Breast cancer forms in the cells of the breast, and it is the most common malignancy during pregnancy [1]. Breast cancer diagnosed during pregnancy or within the first postpartum year is called pregnancy associated breast cancer (PABC) [2]. Approximately one in 3,000 pregnant women are diagnosed with PABCs [1]. PABCs tend to be at an advanced stage when diagnosed and present larger tumors and higher percent of hormone receptor-negative tumors. These cancers are associated with a poor prognosis because metastasis is common. Mechanisms for these tumor growths are being studied and have not been fully understood [2].

The mammary gland is made up of epithelial cells and stromal cells. Stromal cells consist of pre-adipocytes, adipocytes, fibroblasts, blood vessels, inflammatory cells and extracellular matrix. Although breast cancer begins in the epithelium, stromal cells have a role in tumorigenesis [3]. Studies show that the mammary gland development has properties similar to breast cancer, such as reinitiation of cell proliferation, resistance to apoptosis, and angiogenesis, which are properties of tumor progression. The first stage of mammary gland development is called nulliparous. This stage describes women who have not been pregnant and have some epithelial cells filling the gland. During pregnancy, the second stage of development, there is epithelial cell proliferation. During lactation, these epithelial cells expand and differentiate into secretory, milk producing lobular alveoli. At this time the adipocytes decrease their lipid content in the gland. Then, during the involution stage, the secretory epithelium is removed by processes such as apoptosis. Lastly, the adipocytes refill with lipid in the regressed gland similarly to the nulliparous stage.

Changes in the microenvironments of mammary gland development are tumor promoting, allowing for the high mortality rate [4]. The microenvironments from different stages of mammary gland development differ in promoting tumors. For example, remodeling during lactation and involution stages has been specifically implicated in promoting PABCs [4,5].

Lactation, a stage associated with epithelial differentiation and milk production promotes changes in vasculature and adipose tissue [5]. In that study, tumors from pregnant and lactating stages were larger than that in nulliparous and involution stages. The study notes that an increase in hormone levels can be causing the accelerated tumor growth during these stages. However, it suggests that changes in cellular composition within the lactating mammary gland microenvironment could be also accountable for these tumor growths. Through the study, it was found that adipocyte stromal cells (ASCs) from the lactating stage (ASC-Ls) of the mammary gland were responsible for the accelerated tumor growth. The study showed that under identical conditions, ASC-Ls did not accumulate lipid, unlike ASCs of nulliparous, pregnant, involuting, and regressed glands. ASC-Ls were found to be lipolytic, unable to accumulate lipid, and unable to fully differentiate. However, these cells accelerated tumor growth through angiogenesis, the development of new blood vessels.

Cellular retinoic acid binding protein 1 (*Crabp1*) gene was one of the most differentially expressed in ASC-Ls [5]. The inhibition of this gene caused increased lipid accumulation and decreased tumor promotion, indicating that Crabp1 modulates the tumor-promoting environment of ASC-Ls. Crabp1 also affects the tumor promoting activity of carcinoma associated fibroblasts (CAFs), cells surrounding tumors in human patients. Crabp1 could also modulate tumor angiogenesis in CAFs. The ASCs and their expression of Crabp1 seemed to be restricted to lactation and cancer.

High levels of Crabp1 are associated with a poor prognosis of breast cancer [6]. Crabp1, like Crabp2 and Fabp5 (other retinoic acid binding proteins) affects retinoic acid induced cell growth. Crabp1 inhibits retinoic acid (RA) signaling in breast cancer cells. This has been analyzed in breast tumor tissues. These findings on Crabp1 attribute this gene with a role in breast tumor growth. Crabp1 is key in regulation of breast cancer cell response to RA.

Crabp1 and Crabp2 have been highly conserved during evolution. These regulate the transport and metabolism of retinoic acid. Crabp1 keeps RA in the cytoplasm, thereby preventing it from activating gene transcription. There are two types of RA receptors, retinoic acid receptors (RARs) and retinoid x receptors (RXRs). In the presence of RA these can activate or suppress the transcription of genes that have a retinoic acid response element (RARE) in their promoter region. Low levels of Crabp1 expression cause higher levels of RA. Thus, this suggests that Crabp1 limits the availability of RA to the nuclear receptors at the cytoplasm. This works in contrast to Crabp2, whose expression is induced with addition of RA. The regulation of these transcription factors is important for gene activation in cellular differentiation [7-8].

Other research has studied the functional differences between Crabp1 and Crabp2 [9]. CRABPs solubilize and protect the ligand and transport RA in the cell. Crabp2 directly interacts with RAR, which allows for the RAR-RA complex. Crabp1 regulates its ligand by affecting enzymes that metabolize RA. Thus, it might only affect the transcriptional activity of RA if the cell expresses these enzymes.

The regulation of Crabp1 during adipocyte differentiation has also been studied [10]. One of the factors that is involved in transcriptional regulation of Crabp1 is thyroid hormone (T3). The receptor interacting protein 140 (RIP140) has a role in the T3 repression of Crabp1 during

adipocyte differentiation. Crabp1 regulates intracellular concentration of RA and RA is thought to be important for the differentiation of adipocytes.

Two proposed roles of Crabp1 are the retention of RA in the cytoplasm and RA metabolism into its derivatives [11]. Thus, Crabp1 is able to regulate the concentration of active RA able to enter the nucleus for transcriptional regulation. Cells that are sensitive to RA have specific regulations over Crabp1. T3 hormones have been found to regulate Crabp1 by activating it in pre-differentiated cells but suppressing it in cells committed to adipocyte differentiation. In this way T3 can stimulate adipocyte differentiation. On the contrary, RA is known to suppress early adipocyte differentiation. Research has found that if low levels of Crabp1 are present, RA can inhibit cell proliferation of precursor cells. Furthermore, in cells already committed to differentiation, RA in the absence of Crabp1 can facilitate differentiation.

Retinoids, derivatives of vitamin A or retinol, affect growth control, epithelial differentiation and embryonic development [12]. Retinoic acid turns a cell line into differentiated non-proliferating cells. Retinoic acid receptors are transcription factors that bind to the promoters of their target genes and activate or repress transcription depending on the presence of a hormone. Retinoids have antitumor activity and have been implicated in human cancer. The pathway for RA metabolism is a feedback system of genes activated by presence of RA. Retinoid receptors bind DNA on retinoic acid responsive elements (RARE). Retinoic acid treatments have been used against cancers since administration of retinoids has favorable impacts on tumor cells.

Retinoids can affect cells nearby and function intercellularly. These can regulate cell proliferation, differentiation and apoptosis [13]. To activate transcription in stem cells, RA inside the cell is first bound to Crabp2 and transported to the nucleus. Then, it binds to RARs, which bind to RXRs. This complex is bound to DNA and activates transcription of RA primary

response genes. The target genes have enhancers, which contain RAREs to which the RAR-RXR complex can bind. This process induces epigenetic changes, activates transcription of primary target genes, and induces transcription of genes, which further modify gene expression, among other functions. This early signaling cascade can also lead to the differentiation of stem cells. Because of its ability to signal cell differentiation and change patterns of gene expression in tumor cells, retinoids have been a component of cancer treatments. RA is also a potential agent for breast cancer treatment.

Retinoic acid has been shown to affect body fat and insulin sensitivity [14]. Retinoids have a role in controlling lipid and energy metabolism. RA impacts processes that affect mammalian adiposity such as adipogenesis, when pre-adipocytes become adipocytes, and lipogenesis, the process of fatty acid and triglyceride synthesis. Pathways dependent on RAR, such as that of RA, regulate genes encoding proteins of lipid metabolism at the transcriptional level. In humans, an inverse relationship has been noted between vitamin A (from which RA is derived) intake and adiposity. This is because retinoids, their binding proteins, and metabolizing enzymes play a role in the development and metabolic regulation of adipose tissues.

Fatty acid synthase (FAS) is an enzyme that catalyzes the synthesis of fatty acids. Research shows how cellular sterols regulate the *FAS* promoter [15]. Furthermore, sterol regulation is mediated by binding sites for sterol regulatory element binding protein (SREBP) and the transcription factor Sp1. These insights on *FAS* promoter can be used to produce a plasmid with a *FAS* promoter, in order to study the mechanisms that drive *FAS* gene expression.

Transcriptional regulatory mechanisms of *FAS*, specifically in the lactating mammary gland in goats have been studied [16]. Research provides a comparison for human, rats, and goat FAS promoter sequence. Studies characterized the goat *FAS* promoter region. The 5’ flanking

region of the goat *FAS* gene was isolated. The core region of the promoter was found to be conserved in humans, rats, goats, and ruminants. This region has transcription factor binding sites such as Sp1, NF-Y, and two sterol response element (SRE) sites. It was shown that two DNA binding sites for SREBP-1 are required to activate the *FAS* promoter. This suggested the binding of two sterol response elements to the *FAS* promoter as the SREBP-1 regulation mechanism of FAS levels.

Fatty acid synthase plays a central role in lipogenesis in mammals. Researchers have studied the role of sterol regulatory element binding protein (SREBP) in the regulation of *FAS* promoter in vivo [17]. Findings showed that SREBP activates the *FAS* promoter. This study also suggests a role of SRE in regulation of *FAS* gene since it is highly conserved in rat, human, and goose *FAS* promoter sequences.

My research builds on these findings and attempts to merge the two known functions of Crabp1: Does Crabp1 regulate lipid accumulation through its ability to regulate the RA dependent gene *FAS*? We hypothesized that Crabp1 regulates the gene expression of *FAS* in mouse mammary gland adipose stromal cells.

Methods:

Cell Culture and Reagents

Mammary adipose stromal cells (ASCs) were isolated from nulliparous, lactating, and pregnant female mice [4]. ASCs were resuspended in DMEM and plated with 10% fetal bovine serum (FBS) and 1% antibiotic antimyotic (Ab/Am) (Thermo Fisher Scientific). For splitting into a new flask, they were first washed with PBS, and trypsin was used to remove the cells from the culture surface. The 293T cell line was also cultured in DMEM with 10% FBS and 1% Ab/Am. FAS promoter reporter vector was obtained from Genecopoeia. This vector contains -293 to -14 base pairs of the FAS human promoter [16]. Retinoic acid was purchased from Sigma.

FAS and Crabp1 Virus Infection

Primary cells are difficult to transfect, therefore, the 293T cell line was used for transduction of the virus into the primary cells. 2.5 x 10^5 293T cells were plated in a six well plate. 24 hours after plating, the cells were fed with fresh media. Then, Opti-MEM, 7.5µL TransIT-LT1 reagent (Mirus Bio), 600ng target DNA (FAS or FAS and shCrabp1), 400ng delta VPR8.2, and 200ng VSVG were added per well, for a total volume of 250µL. Two wells were used as canary wells and only received 242.5µL Opti-MEM and 7.5µL TransIT-LT1.

24 hours after transfection, 1.5 x 10^5 ASC-N s and ASC-Ls were plated into a six well plate. 48 hours after transfection, the media from the 293T cells were filtered through a 0.45µM filter and added to ASC-Ns and ASC-Ls along with 8µg/mL polybrene. Cells were incubated for four hours at 37°C after which the primary cells were fed with fresh media. 24 hours after virus infection, steps from the previous 24 hours were repeated for a double infection. Three days later the ASC-Ns and ASC-Ls were moved to a 100mm plate and 1µg/mL puromycin was added. The media was changed every two days and 1µg/mL puromycin was added.

Retinoic Acid Treatment

A stock of 0.1M Retinoic acid (R2625 Sigma) was prepared in DMSO and stored at -80°C. ASC-Ns were treated with 0.1μM, 0.5μM, and 1μM RA. For –RA cells were treated with 2μL DMSO as vehicle control. A concentration of 1μM RA was used for all following experiments.

Luciferase Assay

1000 cells per well of the ASC-N-*FAS* promoter and 6 x 10^3 cells per well ASC-L-low Crabp1-*FAS* promoter were plated in a 96 well plate. ASC-N-*FAS* promoter were treated with 1μM RA for 24hrs, 3days, or 8days. The ASC-L-low Crabp1-*FAS* promoter were treated with 1μM RA for 24 hours. The luminescence was measured as described in the Secrete-Pair Luminescence Assay Kit protocol (Genecopoeia) using a Luminometer (Promega). The GL-S reading was normalized to the SEAP reading in order to detect the fold change in *FAS* promoter activity.

Adipocyte Differentiation Assays

For adipocyte differentiation assays, ASCs were plated in six well plates: 1.6 x 10^5 ASC-N, 8.3 x 10^4 ASC-P, and 5.6 x 10^4 ASC-Ls per well. There were allowed to grow to confluence in DMEM supplemented with 10%FBS and 1%Ab/Am. Once confluence was reached, cells were treated with differentiation media: DMEM, 10%FBS, 1% Ab/Am, 0.5mM IBMX(Sigma), 0.1 μM dexamethasone (Sigma) and 0.5 μg/mL insulin (Sigma) and 1μM RA for 14 days. Cells were stained with Oil Red O as follows: cells were rinsed with PBS twice, and then were fixed in 1ml/well 10% formalin/PBS for one hour at room temperature. The wells were rinsed twice with dH_2O and stained with 1ml per well Oil Red O for two hours. The cells were then washed with dH_2O fifteen times. Then, the cells were washed with 1ml per well isopropanol and 300μL of

each well was transferred to a 96-well plate and absorbance was measured at 515nm with an absorbance microplate reader (BioTek ELx808).

RT-PCR analysis

8.75 x 10^5 ASC-Ns were plated in each of four 100mm plates. These were treated with 0.1μL, 0.5μL, and 1μM RA and collected at 24 hours. 1.6 x 10^5 ASC-N , 8.3 x 10^4 ASC-P, and 5.6 x 10^4 ASC-Ls were plated in 6 well plates. ASC-N, ASC-P, and ASC-L were treated with 1μM RA and adipocyte differentiation media (see above) or normal growth media for 14 days. Cell pellets were collected and total RNA was isolated and purified following the RNeasy mini kit (Quiagen). RNA was reverse transcribed to cDNA using an iScript cDNA synthesis kit (Bio-Rad). Quantitative real-time PCR analysis was performed using SYBR Green (Bio-Rad) and a Real-Time PCR system (Bio Rad). Data was analyzed via the delta delta Ct method [18].

Results:

Retinoic acid treatment decreases *FAS* promoter activity of ASC-L-low Crabp1. To determine the effects of RA treatment on *FAS* promoter activity in stromal cells with low levels of Crabp1 we performed a luciferase assay. ASC-N, stromal cells isolated from nulliparous mice, have naturally low levels of Crabp1 protein, while ASC-L-low Crabp1 are stromal cells isolated during lactation that have been treated with shRNA against Crabp1 to reduce Crabp1 protein levels [5]. RA treatment did not appear to change the activity of the *FAS* promoter in ASC-Ns at days 1, 3, and 8 post treatment (Figure 1a). It appears that there is a slight decrease at day 8, however this experiment would need to be repeated to verify if it is a significant decrease. A luciferase assay was also performed to determine *FAS* promoter activity in ASC-L-low Crabp1. Results indicate that RA treatment led to an approximate 22% decrease in *FAS* promoter activity 24 hours after transfection (Figure 1b). Taken together our data suggests that RA does not regulate *FAS* promoter activity in ASC-N but may regulate promoter activity in ASC-L-low Crabp1. It should be noted that this experiment was only conducted one time and needs to be repeated for statistical significance.

Retinoic Acid treatment decreases *FAS* and *Crabp1* mRNA levels in ASC-Ns. RT-PCR was done to measure mRNA levels in ASC-Ns treated with 1uM RA (Figure 1c). Since Crabp1 functions to bind RA we hypothesized that treating ASC-Ns with RA might lead to an increase in *Crabp1* mRNA. By extension if Crabp1 is negatively regulating *FAS* transcription that would lead to a subsequent decrease in *FAS* mRNA levels. Our data indicate that *FAS* mRNA levels decrease by 40% when cells were treated with RA compared to that of untreated cells. However, *Crabp1* mRNA levels also decreased by 26% in cells treated with RA. We then speculated that perhaps *FAS* levels may not be changing but rather its function in lipid metabolism was

decreased. To determine if RA was affecting proteins involved in lipid metabolism we examined mRNA levels of *KLF2* and *Pref-1*. These proteins inhibit adipocyte differentiation and therefore are expected to decrease upon lipid accumulation in mature adipocytes. FAS may function to decrease the levels of these proteins. RT-PCR data indicate that mRNA levels of *KLF2* increased 141% and mRNA levels of *Pref-1* increased 150% in ASC-Ns treated with RA compared to untreated cells. These data suggest that RA is keeping the cells in an undifferentiated state and they are not capable of accumulating lipid. This is in keeping with the decrease in *FAS* mRNA. Taken together the data suggest that RA inhibits lipid metabolism in ASC-Ns but not through an increase in Crabp1. It should be noted that this experiment was only conducted one time and needs to be repeated for statistical significance.

ASC-Ls prevent lipid accumulation even in the presence of adipocyte differentiation media. A lipid accumulation assay was done in ASC-Ns, ASC-Ls, and ASC-Ps treated with media that contains components designed to force adipocyte differentiation and lipid accumulation. Previous data has shown that ASC-Ls accumulate significantly less lipid than ASC-N and ASC-P [5]. We hypothesized that this decrease in lipid accumulation could be due to high levels of Crabp1 inhibiting FAS levels. To determine if the levels of lipid accumulation with RA treatment correspond with the levels of FAS and Crabp1 we performed adipocyte differentiation assays followed by Oil Red O staining. Our results indicate that ASC -Ns treated with RA had increased lipid accumulation by 2 fold compared to untreated cells. Interestingly, ASC-Ps treated with RA showed a decrease in lipid accumulation by 0.5 fold. ASC-Ls showed a slight increase in lipid accumulation. However, this increase in lipid accumulation should be taken in context with the overall amount of lipid accumulation in other cell types. ASC-Ns and ASC-Ps accumulated a much higher amount of lipid when compared to ASC-Ls (Figure 2). These data

correspond to previously published results in that ASC-Ls accumulate less lipid [5] and extends the research by showing that RA treatment increases lipid accumulation levels.

***Crabp1* mRNA levels are correlated with FAS mRNA levels upon retinoic acid treatment in cells forced to accumulate lipid.** To evaluate the gene expression changes in ASCs isolated from multiple stages of development (ASC-pregnant, ASC-N, ASC-L) that underwent the lipid accumulation assay (Figure 2) we extracted the RNA from them at the end of the assay. We hypothesized that changes in Crabp1 might regulate FAS levels which may then contribute to the cells ability to accumulate lipid. This is different from the experiments described above (Figure 1c) in that these cells were provided reagents that force lipid accumulation. It could be that Crabp1 only works to regulate FAS under those conditions. To test this hypothesis we treated cells with RA and adipocyte differentiation media and performed RT-PCR (Figure 3a-d).

Treatment of RA in cells under differentiation conditions increased *Crabp1* mRNA 2.7 fold in ASC-Ns and 14.7 fold in ASC-Ls, but decreased 0.2 fold (80%) in ASC-Ps (Figure 3a). *Crabp2* mRNA increased 2 fold in ASC-Ns, but decreased by 0.06 fold (94%) in ASC-Ps and 0.8 fold (20%) in ASC-Ls (Figure 3b). This shows an inverse relationship between levels of Crabp1 and Crabp2 in ASC-Ls as expected due to their opposite functions in the cell [7]. RA treatment caused *FAS* mRNA levels to increase 1.4 fold in ASC-Ns and 3 fold in ASC-Ls, but a decrease by 0.07 fold (93%) in ASC-Ps (Figure 3c). This suggests that Crabp1 does not regulate *FAS* mRNA levels in cells forced to undergo lipid accumulation.

Although our data do not implicate Crabp1 as a negative regulator of FAS levels, it could be that Crabp1 is regulating FABP4 levels. One of the proteins involved in the adipocyte differentiation pathway is the receptor PPARγ. The binding of another protein called TRAP220 leads to the transcription of *FABP4* and this promotes differentiation of adipocytes. We predicted

that Crabp1 could be involved in this pathway to regulate lipid accumulation in ASC-Ls because it also interacts with TRAP220 [20]. To determine if Crabp1 regulated FABP4, we measured *FABP4* mRNA levels in ASC-Ns treated with both RA and adipocyte differentiation media. Levels of *FABP4* mRNA levels increased 1.4 fold in ASC-Ns and 5 fold in ASC-Ls, but decreased by 0.08 fold in ASC-Ps (Figure 3d). Taken together, our data suggest that the stages of the mammary gland respond to RA treatment differently when exposed to adipocyte differentiation media. In addition, our data suggest that Crabp1 does not negatively regulate *FAS* levels. Increased levels of *FABP4* and *FAS* in ASC-Ls also suggest that high levels of Crabp1 will not necessarily lead to a decrease in lipid synthesis. It should be noted that this experiment was only conducted one time and needs to be repeated for statistical significance.

Discussion:

High levels of Crabp1 in the mammary gland are associated with poor prognosis in PABCs. Previous research indicates that Crabp1 promotes tumor growth and that it prevents lipid accumulation. However it is unclear how Crabp1 prevents lipid accumulation. This study was conducted to begin to understand this mechanism. In this report we have shown that treating cells with RA leads to a decrease in *FAS* promoter activity of ASC-L-low Crabp1, but does not appear to change *FAS* promoter activity in ASC-Ns. It is interesting to note that treatment of ASC-L-low Crabp1 cells with RA decreased the *FAS* promoter activity by 22%. It would be worthwhile to return to those cells to ensure that Crabp1 levels are in fact still low and do not have restored levels of Crabp1. If this was the case we are tempted to speculate that Crabp1 may be regulating the *FAS* promoter. Additional experiments would be needed to ensure the Crabp1 levels as well as performing luciferase assays with ASC-Ls to measure *FAS* promoter activity in cells that normally have high levels of Crabp1.

We also report that retinoic acid treatment also leads to decreased *FAS* and *Crabp1* mRNA levels in ASC-Ns, suggesting that RA inhibits lipid metabolism, but not through increasing Crabp1. However, ASC-Ls (cells with normally high Crabp1 levels) showed a slight increase in lipid accumulation when treated with RA, but extremely low lipid accumulation compared to other cell types, corroborating previously published research [5]. ASC-Ns (cells with low Crabp1) had increased lipid accumulation when treated with RA. These results are consistent with previous research and suggest that the role of Crabp1 binding to RA may not be involved in its ability to regulate lipid accumulation. Furthermore, *Crabp1* mRNA levels correlated with *FAS* mRNA levels upon RA treatment in cells forced to accumulate lipid, suggesting that Crabp1 is not a negative regulator of FAS levels under those conditions.

The data reported herein do not support the hypothesis that Crabp1 regulates lipid accumulation through the regulation of the RA dependent gene *FAS*. It is possible that Crabp1 is able to regulate lipid accumulation independent of its ability to bind to RA, possibly through different domains on the protein. This is a plausible explanation for why RA dependent genes were affected by RA treatment, but not in a Crabp1 dependent matter. It should be noted that RA has been shown to inhibit lipid metabolism and adipogenesis [19]. Others studies have shown that RA does not decrease lipid accumulation [22]. Therefore lipid metabolism and lipid accumulation could involve distinct pathways and Crabp1 could be acting solely to regulate lipid accumulation. Crabp1 binding and retention of RA would therefore not affect FAS and FABP4 levels. However, whether or not Crabp1 inhibits lipid accumulation through regulation of RA still needs to be fully determined.

In order to fully address the hypothesis we must first return to the functions of Crabp1. It is known to bind and sequester RA in the cytoplasm and thereby prevent transcriptional activation of RA responsive genes [6]. It also functions to prevent lipid accumulation [5]. Rather than try to determine if Crabp1 regulates lipid accumulation by binding to RA it would first be important to determine how Crabp1 accomplishes these two functions. Crabp1 binds to RA at the lipocalin/ cytosolic fatty-acid binding protein family domain [21]. However the domain(s) responsible for the lipid accumulation function are unknown. It would be interesting to perform experiments that determine the structure-function relationship of the protein by overexpressing truncated protein and performing lipid accumulation assays. This would be the next step in our understanding of the role of Crabp1 in lipid accumulation as well as if this function is important in its role as a tumor promoter.

It is important that the known functions of Crabp1 in the cell be understood in order to study how Crabp1 promotes tumor growth, particularly in ASC-Ls. Research has shown that PABCs diagnosed during lactation are associated with poor prognosis [2,5]. Furthermore, high levels of Crabp1 are associated with poor prognosis in breast cancer [6]. Knowing the RA and lipid associated functions of Crabp1, and that it plays a role in tumor promotion in PABCs, led us to investigate its role in FAS expression in the presence of RA treatment. Although our results suggest that Crabp1 is not regulating FAS expression, these are consistent with previous research in that ASC-Ls fail to accumulate lipid in comparison to other cell types. Also, our results suggest that RA increases lipid accumulation in ASC-Ns and slightly on ASC-Ls, but not ASC-Ps, thus it could be dependent upon the microenvironment of the mammary gland at particular stages. It is probable that high levels of Crabp1 in ASC-Ls decreased the impact of this RA function. However, further understanding of Crabp1 structure-function relation is needed to investigate its lipid regulation function. The finding of mechanisms present during this stage of mammary gland development and how these can promote tumorigenesis, could allow us to further understand and treat PABCs.

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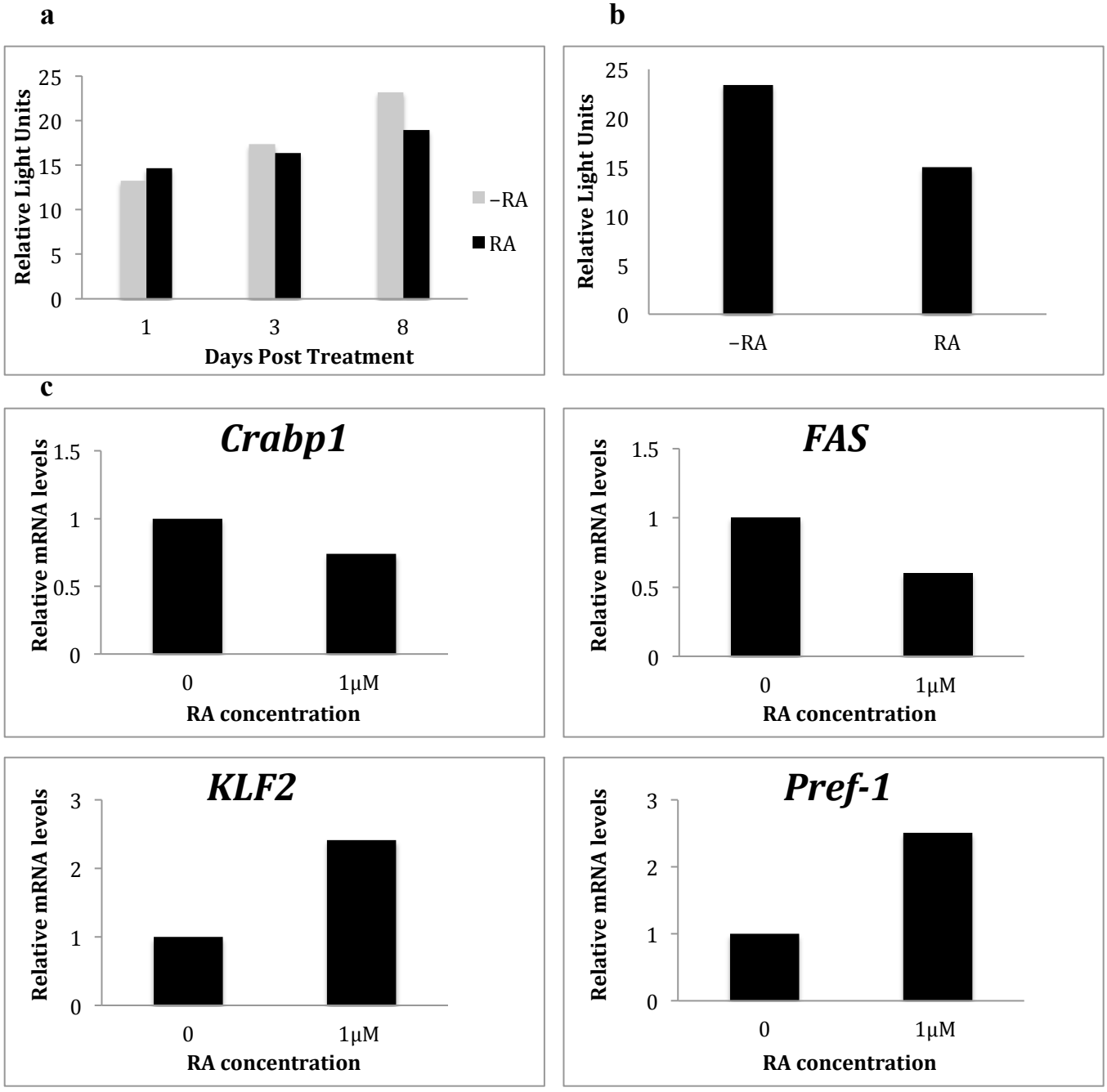

Figure 1: Retinoic acid treatment decreases *FAS* promoter activity of ASC-L-low Crabp1 and decreases *FAS* and *Crabp1* mRNA levels in ASC-Ns. (a) Luciferase assay of ASC-Ns transfected with the *FAS* promoter. These were treated with 1μM RA and were collected at 24hrs, 3 days, and 8 days. Treated cells were compared to untreated cells (-RA). **(b)** ASC-L-low Crabp1 cells transfected with the *FAS* promoter were treated with 1μM RA. RA treated ASC-L-low Crabp1 were compared to untreated cells. **(c)** RT-PCR was used to measure mRNA levels of several genes in transfected ASC-Ns expressing the *FAS* promoter. Data was normalized to GAPDH. RA treated cells were compared to untreated cells. Untreated cells were set to one (n=1).

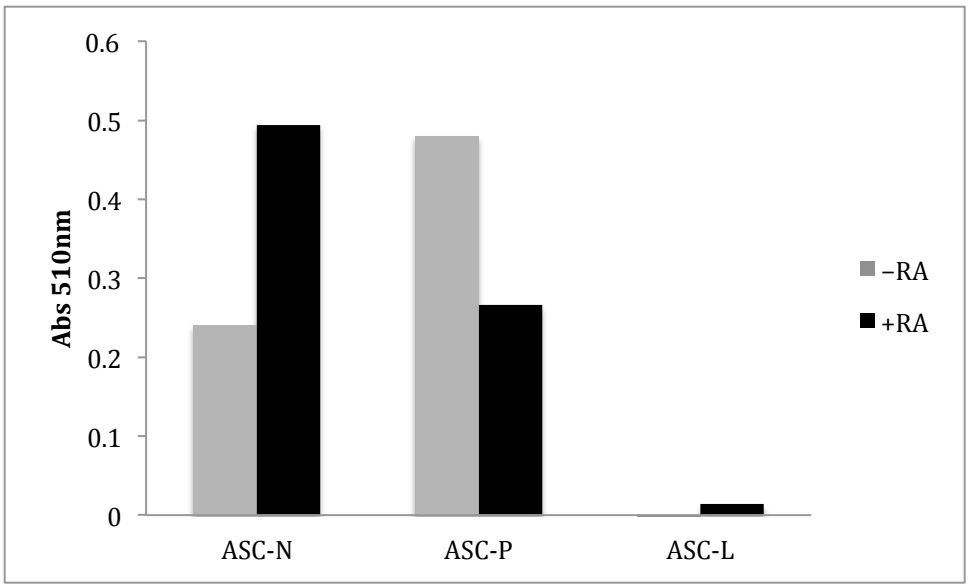

Figure 2: Retinoic acid treatment effects on lipid accumulation are stage dependent. Quantification of Oil Red O staining of ASC-Ns, ASC-Ps, and ASC-Ls treated with adipocyte media and 1μM RA. Absorbance was measured at 510nm. RA treated cells are compared to untreated cells (-RA). (n=1).

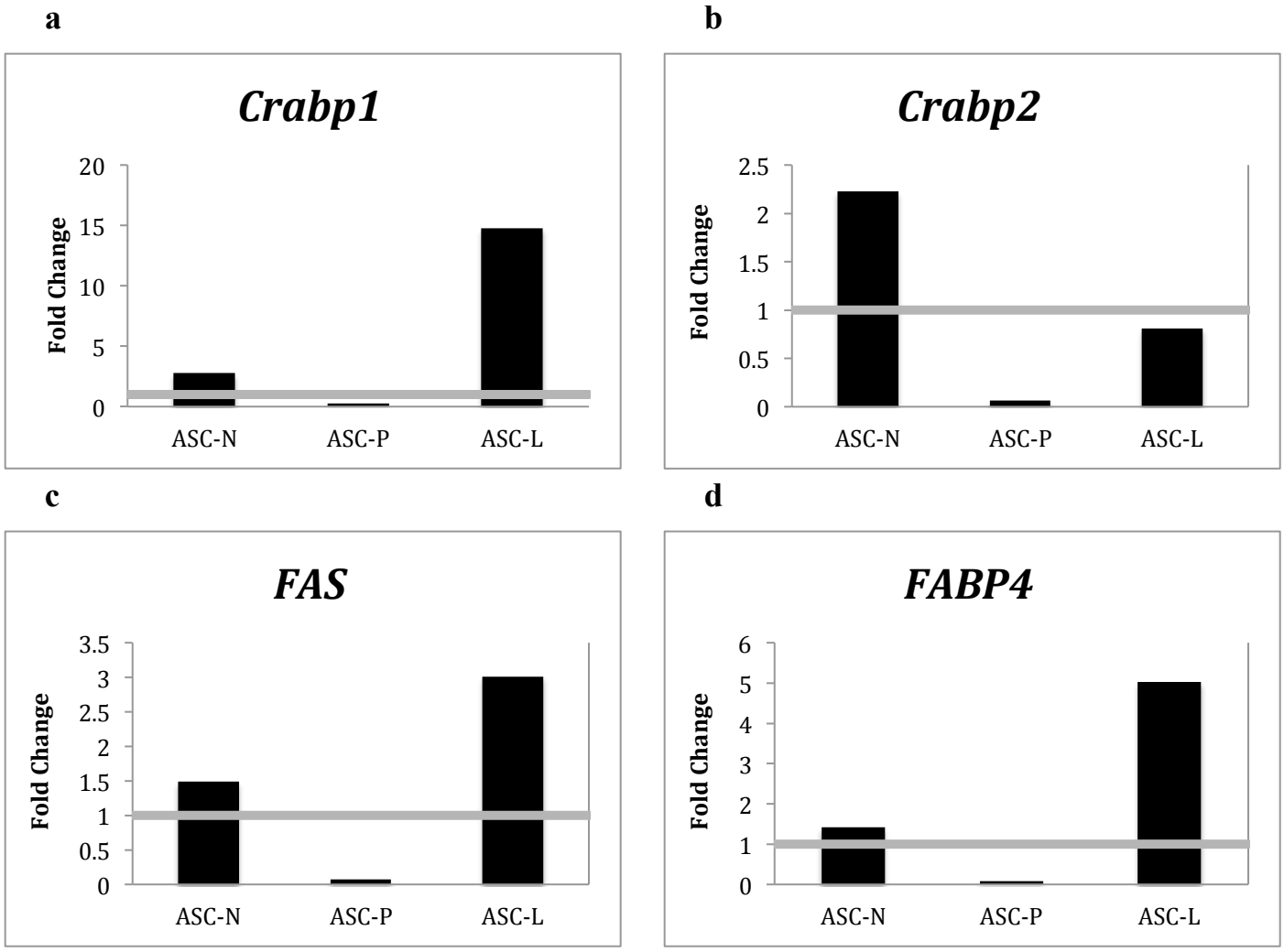

Figure 3: Retinoic acid treatment leads to different levels of gene expression dependent on the developmental stage of the cells. (a-d) RT-PCR using ASC-N, ASC-P, and ASC-Ls treated with adipocyte differentiation media and 1μM RA. Data was normalized to GAPDH in the presence of adipocyte media and 1μM RA and is represented as fold change in comparison to untreated (-RA) cells. The horizontal line indicates untreated cells set to one. Bars above the line indicate an increase in expression and bars below the line indicate a decrease in expression compared to untreated cells. (n=1).