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13. ABSTRACT (Maximum 200 Words)

Recent evidence has suggested that cross-talk between the TGFβ and ER signaling pathways exist. Data from our laboratory suggests that although a physical interaction between ER and Smad3, a downstream signaling protein of the TGFβ pathway, does not exist, a functional interaction is present. ERα and ERβ inhibit Smad3 transcription on the TGFβ reporter plasmid p3TP-lux in a ligand-dependent manner and this inhibition may be suppressed and subsequently reversed by the anti-estrogens 4-OH-tamoxifen and ICI 182,780. As both ERα and Smad3 interact with members of the Ap1 family and since the p3TP-lux promoter has three Ap1 binding sites, we next sought to examine whether Ap1 factors may be limiting factors in Smad3 transcription on p3TP-lux. Results suggest that overexpression of c-Jun but not c-Fos was able to reverse the effect of ERα on Smad3 transcription in Cos1 cells. Our results suggest that Ap1 factors may be important in the regulation of TGFβ signalling by ER. Experiments are currently underway to determine if these observations are relevant in a breast tumor cell model.

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Table of Contents

Cover..1

SF 298.................................. 2

Table of Contents................. 3

Introduction..4

Body.............. 5

Key Research Accomplishments.. 7

Reportable Outcomes..8

Conclusions.. 8

References..9

Appendices...11

4. INTRODUCTION

A growing body of evidence has begun to emerge suggesting a cross-talk between the estrogen receptor (ER) and transforming growth factor-β signaling pathways. Smad3, a downstream signaling protein of the TGFβ cascade has previously been shown to interact with the glucocorticoid (GR)[1], vitamin D (VDR)[3] and androgen (AR)[3,4] receptors. As AR, GR and VDR all belong to the steroid nuclear receptor superfamily, to which the ER also belongs, it is not surprising that soon after Matsuda et al.[5] described a physical as well as functional interaction between the ER and Smad3. According to these authors[5,6], Smad3 interacts with ERα and $ER\beta_1$ in 293T (human embryonic kidney carcinoma cells) and MCF-7 (human breast carcinoma) cells and that such an interaction is dependent on activation of both the estrogen and TGFβ signaling cascades. Furthermore, these authors then proceeded to show that the other receptor Smads, including Smad1 and Smad2, as well as the co-Smad, Smad4, co-immunoprecipitate with ER[6]. As the overall goal of our research was to understand the importance of the interaction between Smad3 and the ER as it pertains to human breast tumorigenesis and breast cancer progression, our first specific aim was to define the specificity of the interaction of Smad3 with the ER family, both *in vitro* and *in vivo* using co-immunoprecipitations. As reported previously in my annual summary report (July 2001-July 2002), our results suggest that, in contrast to that reported by Matsuda et al.[5], Smad3 does not directly interact with neither ERα nor ERβ in Cos1 cells. As a result, we had suggested that cell type specific factors may be influencing the interaction of ER with Smad3.

While no physical interaction between the ER and TGFβ signaling pathways was observed by us, we next wanted to examine whether there was a functional interaction between these two pathways. To examine whether Smad3 affects ER signaling, we utilized the following two ER reporter genes:

- **A. ERE-II-luc**: obtained from Dr. P. Webb (University of California); contains two copies of the vitellogenin A2 estrogen responsive elements upstream of a luciferase gene.
- **B. $TGF\beta_3$-luc**: obtained from Dr. Webb (University of California); contains an estrogen responsive segment from the $TGF\beta_3$ promoter upstream of a luciferase reporter gene.

To examine whether ER affects Smad3 signaling, we utilized the following two TGFβ reporter genes:

- **C. p3TP-lux**: obtained from Dr. Massague (Rockefeller University); a luciferase reporter plasmid that contains a Smad binding element from the plasminogen activator inhibitor 1 (PAI-1) gene downsteam of three TPA-responsive elements of the human collagenase gene.
- **D. Collagen 7(A1) (-524/+92)-luc**: obtained from Alain Mauviel (Paris, France); a luciferase reporter plasmid that contains a segment of the human collagen 7(A1) promoter that contains a Smad binding element upstream of a consensus Ap-1 site.

5. BODY

A) ERE-II-luc and *B) TGFβ_3*

To determine if Smad3 affects ER transcriptional activity, the ERE-II-luc reporter plasmid was transiently transfected into Cos1 cells along with increasing amounts of Smad3, ERα/ERβ_1 and the β-galactosidase reference gene pCH110 in the presence or absence of 10nM estradiol. Our results suggest that no significant difference in luciferase activity was observed between those samples treated with Smad3 and ERα/ERβ_1 to those treated with ERα/ERβ_1 alone. The vitellogenin ERE represents the classical model of ER action, whereby an activated ER dimer binds directly to the ERE to modulate gene transcription. Evidence also suggests, however, that the ER may form protein-protein interactions with other transcription factors that are bound to their cognate DNA binding sites and through this interaction, affect target gene transcription[7,8]. Therefore, we next wanted to examine whether Smad3 could affect ER transcription on such "non-classical" ER promoters, namely the TGFβ_3 promoter[9]. Cos1 cells were transiently transfected with the ER reporter plasmid TGFβ3, along with increasing amounts of Smad3, ERα and pCH110 in the presence or absence of 100nM Ly11078, a raloxifene analog, of which has previously been shown to maximally stimulate TGFβ_3 activity by ER[10]. Like the classical ERE, our results suggest that Smad3 does not affect ER transcription on the TGFβ_3 reporter plasmid. While these results are in disagreement with those of Matsuda et al.[5], who suggest that Smad3 increases ER transcriptional activity on a vitellogenin ERE, our observations are in agreement with those of several other laboratories[3,4]. In the course of these experiments, however, we observed that our internal control, the constitutively active β-galactosidase pCH110, activity increased with increasing amounts of transfected Smad3 plasmid ($p<0.001$), suggesting that Smad3 may increase the activity of this plasmid. Therefore, we tested several alternate internal control plasmids, including the pRL-tk-luc plasmid (Promega), of which we currently use. The pRL-tk-luc does not appear to be affected by Smad3 expression and also has the added advantage of doing dual luciferase assays (one assay for the firefly luciferase and one for the renilla luciferase) that saves time. Although the discrepancy between our results and those of Matsuda et al.[5] are unknown, it may have been due to the different cellular environments in which our experiments were performed. Therefore, we next examined the affect of Smad3 over-expression in our MCF7 human breast cancer cell line, the cell line used by Matsuda et al.[5] While transfection of these cells did prove to be challenging, our results suggest that, similar to Cos1 cells, Smad3 does not affect ER transcription.

C) p3TP-lux

Although Smad3 does not affect ER transcription in Cos1 or MCF7 cells, the question still remained whether ER could affect Smad3 transcriptional activity. We previously had reported in our annual summary that both ERα ($p<0.001$) and ERβ_1 ($p<0.05$) inhibits Smad3 transcription on the p3TP-lux reporter plasmid in Cos1 cells in a ligand dependent manner. We have now assessed the specificity of estrogen effects on

Smad3 transcriptional activity by utilizing the anti-estrogens 4-OH-tamoxifen and ICI 182,780. Our results suggest that the inhibitory effect of ERα and $ER\beta_1$ in the presence of estradiol on p3TP-lux is suppressed and subsequently reversed by both 4-OH-tamoxifen (100nM) (ERα: $p<0.01$; ERβ: $p<0.005$) and ICI 182,780 (500nM) (ERα: $p<0.005$; ERβ: $p<0.01$) suggesting that the effect of ER on Smad3 transcription is ligand dependent. The ERβ variants, $ER\beta_2$ and $ER\beta_5$, do not affect Smad3 transcriptional activity and this may be in part due to their inability to bind ligand[10]. Although we are currently in the process of confirming these results in the ERα positive human breast carcinoma cell line, MCF7, results suggest that ERα inhibits p3TP-lux ($p<0.005$) and that this inhibition may be reversed by the anti-estrogens 4-OH-tamoxifen ($p<0.0002$) and ICI 182,780 ($p<0.0007$).

As described previously, the Smad3 p3TP-lux reporter plasmid consists of the Smad binding element (SBE) from the PAI-1 promoter downstream of three TPA-responsive elements that bind Ap-1 transcription factors. Evidence suggests that both the SBE and the Ap-1 sites are critically important for induction by both TGFβ and Smad3 overexpression[11]. In our Cos1 cells, over-expression of cJun or cFos increases ($p<0.05$) p3TP-lux transcriptional activity above that observed with Smad3 over-expression alone, suggesting that Smad and Ap-1 complexes may synergize to activate TGFβ responsive promoters. Furthermore, others have shown that TGFβ cooperates with cJun/cFos on Ap-1 promoters to enhance transcriptional activity[11,12,13] and this increase is thought to be mediated by an interaction between Smad3 and cJun[12,14]. Interestingly, ERα has also been suggested to interact with cJun but not with cFos in the presence of estradiol[15]. Therefore, we wanted to test whether cJun was a limiting factor in the activation of p3TP-lux in the presence of activated ERα. Cos1 cells were transiently transfected with Smad3, ERα, pCH110, p3TP-lux and increasing amounts of cJun or cFos in the presence or absence of estradiol. In agreement with our hypothesis, cJun was able to reverse the inhibition of activated ERα on p3TP-lux activity ($p<0.05$) while cFos was not. Therefore, our working hypothesis to date is as follows: in the absence of estradiol, ERα is unable to interact with cJun and thus, cJun is free to bind to and activate p3TP-lux. In the presence of estradiol, however, cJun is no longer able to bind DNA as it is sequestered away by activated ERα. As cFos does not appear to interact with ERα, over-expression of cFos is unable to reverse the inhibition of p3TP-lux in the presence of activated ERα.

While this hypothesis fits very well with ERα, the story is not as simple for ERβ. For one, no evidence exists showing an interaction between ERβ and Ap-1 proteins. Secondly, in our experiments over-expressing cJun and cFos in the presence and absence of activated ERβ, we began to observe a ligand-independent inhibition of p3TP-lux activity that we had not seen in our earlier experiments. We are currently in the process of trying to understand the implications of these results to our working hypothesis. Our model of the mechanism by which ER inhibits TGFβ transcription was initially developed in Cos1 cells, or green monkey kidney carcinoma cells. We are currently testing the validity of our model in the ERα positive human breast carcinoma cell line, MCF7. In addition, we also have available to us a stable transfected MCF7 clone that

over-expresses ERα and another clone that over-expresses ERβ in the presence of doxycyclin. Therefore, we are interested in determining if our hypothesis is also true in these two cell lines.

D) Collagen 7(A1) (-524/+92)-luc

Similar to the p3TP-lux reporter plasmid, the collagen 7(A1) promoter is activated by TGFβ and Smad3 over-expression[16]. Although this reporter plasmid contains a SBE upstream of a Ap-1 site, activation of Ap-1 appears to inhibit TGFβ and Smad3 activation[16,17]. Therefore, if our model for the p3TP-lux is also true for the collagen 7(A1) promoter, addition of activated ERα should increase transcriptional activity as the ER would then be removing an inhibitor of transcription. In Cos1 cells transiently transfected with collagen 7(A1) (-524/+92)-luc, pCH110, Smad3 and increasing amounts of ERα in the presence or absence of estradiol, no significant effect of ERα on collagen 7(A1) transcription was observed although there was a general trend for increased luciferase activity in the presence of estradiol and ERα. However, in the presence of estrogen and the anti-estrogens 4-OH-tamoxifen or ICI 182,780 there was a significant ($p<0.01$ and $p<0.0005$, respectively) decrease in collagen 7(A1) activity as compared to that of estradiol alone. Similar results were also obtained for ERβ. Therefore, it appears as though our model of cJun sequestering by ERα may also be true with respect to this promoter. Studies are currently being performed to test this hypothesis in the MCF7 breast cancer cell line.

6. KEY RESEARCH ACCOMPLISHMENTS

- Smad3 does not affect ER transcriptional activity on either the vitellogenin ERE regulated promoter or on the $TGF\beta_3$ promoter in Cos1 cells.
- Smad3 over-expression increases pCH110 (a constitutively active β-galactosidase reporter gene) activity in Cos1 cells.
- ERα and $ER\beta_1$ inhibit Smad3 transcription on p3TP-Lux in a ligand-dependent manner and this effect may be prevented and subsequently reversed by the anti-estrogens 4-OH-tamoxifen and ICI 182,780.
- $ER\beta_2$ and $ER\beta_5$ do not affect Smad3 transcription on the p3TP-lux reporter.
- cJun and cFos over-expression increases p3TP-lux activity in the presence and absence of Smad3.
- cJun but not cFos reverses the effect of ERα on p3TP-lux activity in Cos1 cells.
- Optimization and validation of the Smad3 reporter gene, p3TP-lux, in MCF-7 cells.
- ERα expression inhibits p3TP-lux activity in MCF7 cells and this may be reversed by the anti-estrogen 4-OH-tamoxifen but not by ICI 182,780.
- Collagen 7(A1) activity is not affected by ER levels in Cos1 cells, although its transcription is inhibited by 4-OH-tamoxifen and ICI 182,780.

7. REPORTABLE OUTCOMES

Cherlet T, Murphy LC (2003). Inhibition of TGFβ signaling by estrogen receptors is reversed by cJun but not cFos overexpression. Abstract to be presented at 94th Annual American Association of Cancer Research Meeting, July 11-14, Washington, D.C. **Appendix 1.**

Cherlet T, Murphy LC (2002). Crosstalk between the transforming growth factor-beta and estrogen receptor signaling pathways. Abstract presented at Era of Hope: Department of defense breast cancer research meeting, September 25-28, 2003, Orlando, Florida. **Appendix 2.**

Cherlet T, Murphy LC (2002). Cross-talk between the transforming growth factor beta (TGFbeta) and estrogen receptor (ER) signaling pathways in human breast cancer. Abstract presented at Research Day 2002, June 4-6, 2002, Winnipeg, Manitoba. **Appendix 3.**

8. CONCLUSIONS

Our preliminary experiments together with our current results suggest that a cross-talk between the ER and TGFβ signalling pathways exists. Although a direct physical interaction between Smad3 and ER does not appear to exist in our cell models, a functional interaction between these two pathways does occur. Although increased Smad3 expression does not effect ER transcription, ER is able to repress Smad3 transcriptional activity on the p3TP-lux reporter gene in a ligand dependent manner. The mechanism by which this inhibition occurs appears to involve the Ap-1 family of transcription factors. We are currently investigating our model in a human breast carcinoma cell line.

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Appendix 1

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INHIBITION OF TGFß SIGNALLING BY ESTROGEN RECEPTORS IS REVERSED BY C-JUN BUT NOT C-FOS OVEREXPRESSION

Tracy Cherlet and Leigh Murphy. University of Manitoba, Winnipeg, Manitoba, Canada.

Activation of the TGFβ signalling cascade has been shown to negatively regulate cellular proliferation. However, many breast cancers are resistant to the growth inhibitory effects of TGFβ despite having an apparently intact TGFβ pathway. Smad3, a downstream signalling protein of the TGFβ pathway, has recently been reported to interact with the estrogen receptor (ER) family thereby modulating ER and TGFβ signalling[1]. The ER family consists of two members, ERβ and ERα. In normal breast tissues, the relative expression of ERβ is high while ERα levels are low. During breast tumorigenesis, however, ERβ expression decreases while ERα increases dramatically. Therefore, the cross-talk between the TGFβ and ER pathways may be important in normal breast tissue that is then altered during human breast tumorigenesis. The goal of this study was to further examine the modulation of TGFβ transcriptional activity by ER and the TGFβ effects on ER transcription. Cos1 cells were transiently transfected with either the ER reporter plasmids ERE-II-luc (contains two copies of the vitellogenin A_2 estrogen response elements (EREs)) or $TGF\beta_3$-luc or the

Smad3 reporter plasmid p3TP-lux in addition to Smad3, ER and the constitutive β-galactosidase expression vector pCH110. In contrast to previous findings[1], our results suggest that Smad3 does not affect ERα nor ERβ transcriptional activity on either the ERE-II-luc or the TGFβ_3-luc promoter. However, ERα and ERβ were able to inhibit Smad (p3TP-lux) transcription in a ligand-dependent fashion. This inhibition may be suppressed and subsequently reversed by the anti-estrogens 4OH-tamoxifen and ICI 182,780. The ERβ variants, ER$\beta_{2/cx}$ and ERβ_5, did not affect Smad3 transcription. As both ERα and Smad3 interact with members of the Ap1 family and since the p3TP-lux promoter has three Ap1 binding sites, we next sought to examine whether Ap1 factors may be limiting factors in Smad3 transcription on p3TP-lux. Results suggest that overexpression of c-Jun but not c-Fos was able to reverse the effect of ERα on Smad3 transcription in Cos1 cells. Our results suggest that Ap1 factors may be important in the regulation of TGFβ signalling by ER. As ER expression alters during breast tumorigenesis, the cross-talk between the ER and TGFβ pathways may be altered and therefore, have an important role in human breast tumorigenesis. (1) Matsuda T, Yamamoto Y, Muraguchi A, Saatcioglu F (2001). J. Biol. Chem. 276(46):42908-42914.

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Appendix 2

Era of Hope

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Orange County Convention Center
Orlando, Florida

Proceedings
Volume I

CROSSTALK BETWEEN THE TRANSFORMING GROWTH FACTOR-BETA AND ESTROGEN RECEPTOR SIGNALING PATHWAYS

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The estrogen receptor (ER) family consists of ER-alpha and ER-beta. In normal breast tissues, expression of ER-beta and its variants is relatively high while ER-alpha levels are low. During breast tumorigenesis, however, ER-beta expression decreases while ER-alpha increases. Therefore, ER-beta may play an important role in normal breast tissues that may be altered throughout breast tumorigenesis. As identification of factors that specifically interact with ER-beta may help to define a putative role for ER-beta, we used the ER-beta2 variant as bait in the yeast two-hybrid screen. Preliminary results suggest that ER-beta interacts with Smad3, a downstream signaling protein of the transforming growth factor-beta (TGF-beta) cascade. Activation of the TGF-beta signaling cascade normally negatively regulates cellular proliferation. However, many breast cancers are resistant to the growth inhibition of TGF-beta despite containing all the components necessary for signal propagation. As Smad3 interacts with other members of the steroid nuclear receptor superfamily, cross-talk between the TGF-beta and ER pathways may exist. We hypothesize that ER-beta and/or its variants interact with the TGF-beta signal transduction pathway and that this interaction modulates TGF-beta signaling. Initially, we examined interactions between ER and Smad3 in vitro. ER and Smad3 were radiolabelled using a coupled transcription/translation system and immunoprecipitated. When low levels of ER-alpha were present, an interaction was observed while at high ER-alpha levels, the interaction was abolished. An interaction between ER-beta1 and Smad3 was also observed. Secondly, we examined whether cross-talk between Smad3 and ER alters either Smad3 or ER transcriptional activity. Cos1 transient transfections with an ERE-CAT suggest that Smad3 does not affect ER-alpha transcription. However, ER-alpha and ER-beta inhibited Smad3 (p3TP-luciferase) transcription in a ligand-dependent fashion. As ER isoform expression and TGF-beta activation altered during breast tumorigenesis, cross-talk between these pathways may have a role in breast tumorigenesis.

Army Medical Research and Materiel Command under DAMD17-01-1-0307 supported this work.

Appendix 3

ResearchDay 2002

Bio-Imaging

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CROSS-TALK BETWEEN THE TGF-β AND ER SIGNALING PATHWAYS.

Tracy Cherlet and Leigh Murphy
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The ER family consists of ERα and ERβ. In normal breast tissues, expression of ERβ is high while ERα levels are low. During breast tumorigenesis, however, ERβ expression decreases while ERα increases. Therefore, ERβ may play an important role in normal breast tissues that is altered in breast tumorigenesis. Results from a yeast two-hybrid screen suggest that ERβ interacts with Smad3, a signalling protein of the TGFβ cascade. Although TGFβ normally negatively regulates cellular proliferation, many breast cancers are resistant to TGFβ. As Smad3 interacts with other members of the steroid nuclear superfamily, cross-talk between the TGFβ and ER pathways may exist. We hypothesize that ERβ interacts with the TGFβ pathway and that this interaction modulates TGFβ signaling. Initially, we examined interactions between ER and Smad3 *in vitro*. ER and Smad3 were radiolabelled using a coupled transcription/translation system and immunoprecipitated. When low levels of ERα were present, an interaction was observed while at high ERα levels, the interaction was abolished. An interaction between ERβ and Smad3 was also observed. Secondly, we examined whether cross-talk between Smad3 and ER alters Smad3 or ER activity. Cos1 transient transfections with an ERE-Luc suggest that Smad3 does not affect ERα nor ERβ transcription. However, ERα and ERβ inhibited Smad3 (p3TP-Lux) transcription in a ligand-dependent fashion. As ER expression and TGFβ activation alter during breast tumorigenesis, cross-talk between these pathways may have a role in breast tumorigenesis.