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**4. TITLE AND SUBTITLE**
Interaction Between Estrogen Receptor-β and the Transforming Growth Factor-β Signaling Cascade in Human Breast Tissue

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**13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)**
The overall goal of this research is to understand the importance of the interaction between Smad3 and the estrogen receptor (ER) as it pertains to human breast tumorigenesis and breast cancer progression. Preliminary data from our laboratory had suggested that ERα, ERβ1, ERβ2, and ERβ3 interact with Smad3 in the yeast two-hybrid system. Our current data suggests that a weak interaction between ERα and ERβ1 with Smad3 exists in vitro but not in vivo. Furthermore, although Smad3 does not affect ER transcriptional activity on a vitellogenin ERE, both ERα and ERβ1 inhibit Smad3 transcriptional activity on the p3TP-Lux reporter in Cos1 cells. However, the ERβ variants, ERβ2 and ERβ3, did not affect Smad3 transcription. We are currently in the process of confirming these results in a human breast cancer cell line. Overall, the data support the hypothesis that ER interacts with the TGFβ signal transduction pathway. The possible mechanisms by which ER affects Smad3 transcription are being investigated.

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4. BODY

The overall goal of this research is to understand the importance of the interaction between Smad3 and the estrogen receptor (ER) as it pertains to human breast tumorigenesis and breast cancer progression. The ER family consists of the classical ER, ERα, and the recently described ERβ. Although the definitive roles of ERα in the development and progression of breast cancer have been partially elucidated, the role of ERβ remains unknown. However, ERβ and ERβ variant mRNA and proteins have been identified in breast cancer cell lines as well as in both normal and neoplastic human breast tissues\(^1\)\(^,\)\(^2\)\(^,\)\(^3\)\(^,\)\(^4\)\(^,\)\(^5\)\(^,\)\(^6\). In normal breast tissue, expression of ERβ is more frequently and likely higher compared to ERα while during breast tumorigenesis, the relative expression of ERβ:ERα decreases\(^5\). Therefore, ERβ and its variants may play an important role in normal breast tissues that is altered in breast tumorigenesis and the identification of factors that interact with ER may help to identify a role for ERβ. Preliminary experiments from our laboratory using the yeast two-hybrid system suggested that an interaction between ERα, ERβ\(_1\), and ERβ\(_2\) with Smad3 may exist\(^7\). Smad3 is a downstream signaling protein of the transforming growth factor β (TGFβ) signaling cascade that has previously been shown to interact with the androgen (AR)\(^8\)\(^,\)\(^9\), glucocorticoid (GR)\(^10\) and vitamin D (VDR)\(^11\) receptors. AR, GR and VDR all belong to the steroid nuclear receptor superfamily to which the ER also belongs, further suggesting that a cross-talk between ER and TGFβ may occur. Therefore, we proposed to test the hypothesis that ERβ and/or its variants directly interact with the TGFβ signal transduction pathway and is part of the mechanism through which ERβ and/or its variants negatively modulate the ERα proliferative signal transduction pathway.

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 (co-IPs). We have cloned the full length open-reading frame of ERα, ERβ\(_1\), ERβ\(_2\), and ERβ\(_3\) in frame with the N-terminal 6 x histidine and xpress tagged expression vector pcDNA4 (Invitrogen) and confirmed the constructs to be in-frame. Earlier last year we were kindly given Smad2, 3 and 4 expression plasmids from Dr. Attisano (University of Toronto) and these have also been confirmed by sequencing. To determine whether an ER/Smad3 interaction occurs in vitro, we in vitro transcribed/translated S\(^35\) radiolabelled ER and Smad3 using the TnT coupled reticulocyte lysate system (Promega). Proteins were mixed on ice in the presence or absence of 10nM estradiol and immunoprecipitated (IP) with either ERα, His (recognizes the histidine residues on the tagged ER) or β-galactosidase (negative control). IPs with a Smad3 specific antibody were not performed in vitro as this antibody was raised in rabbits and our reticulocyte lysate in which we produce our proteins is also rabbit. IP products were run on a 10% SDS-PAGE gel and visualized by autoradiography. Our results suggest that an interaction between ERα and ERβ\(_1\) with Smad3 may exist, although the interaction appears to be weak. No interaction between the ERβ variants, ERβ\(_2\) and ERβ\(_3\), with Smad3 occurred in vitro under these conditions. Several attempts were also made to determine the interaction in vivo in Cos1 cells. Cells were transfected with ER and Smad3 in the presence or absence of 10nM
estradiol and subsequently the cell lysates were IP for either ER or Smad3. Results from these experiments suggested that an interaction between ERα and ERβ1 with Smad3 did not occur in vivo in Cos1 cells or that the interaction was too weak to be detected using the IP methodology. Recently however, Matsuda et al. described the physical interaction between Smad3 and ER. Through a series of co-IPs, these authors suggest that Smad3 interacts with ERα and ERβ1 in 293T and MCF-7 cells and that this interaction is dependent on activation of both the estrogen and TGFβ signaling cascades. Further studies have shown that ERα and ERβ1 interact with Smads 1, 2, 3 and 4 upon stimulation of the ER and TGFβ pathways. Therefore, cell type specific factors may influence the interaction of ERs and Smad3.

In our original research proposal, we next wanted to examine the structural/functional regions of ER and Smad3 that are required for binding. Although Matsuda et al. do not describe the region of the ER that specifically binds Smad3, they do demonstrate that the DNA binding domain of ER is involved in its interaction with Smad1. In addition, these authors have shown that the MH2 domain of Smad3 binds ER.

We next wanted to determine whether the interaction between Smad3 and ER affects ER transcriptional activity. We have obtained a vitellogenin estrogen responsive (ERE) luciferase reporter plasmid from the laboratory of Dr. Webb (University of California). To ensure that the plasmid was indeed ER responsive, we transiently transfected Cos1 cells with the ERE, ERα and the β-galactosidase reference gene pCH110, in the presence or absence of 10nM estradiol. Indeed, results indicate that upon estradiol stimulation, there was a 7 fold increase in luciferase activity. When increasing amounts of Smad3 were co-transfected into Cos1 cells with the ERE, ERα and pCH110 in the presence or absence of 10nM estradiol, no significant difference in luciferase activity between samples treated with Smad3 and ERα compared to those treated with ERα alone was observed. Similar results were also obtained when ERβ1 was transfected rather than ERα. As data from our laboratory suggests that the ERβ variants do not have transcriptional activity of their own, ERE-reporter genes have not been used in conjunction with the variants. Our observation that Smad3 does not affect ER transcription on the vitellogenin ERE is in agreement with several other laboratories. However, Matsuda et al. suggest that Smad3 increases ER transcriptional activity. Although the discrepancy between these results is unknown, it may be due to the different cellular environments in which the experiments were performed or to differences in reporter genes. We are currently in the process of confirming our results in the T5 human breast cancer cell line which contains endogenous ERα and are TGFβ responsive. However, these cells have proven to be extremely difficult to transiently transfect and we are currently testing several transfection methods to overcome this hurdle. The vitellogenin ERE-reporter plasmid described above represents a classical ERE, in which ER directly binds to the DNA. However, ER has also been shown to regulate target gene transcription in a non-classical manner, in which the ER interacts with other proteins (i.e. c-Jun) that then bind to DNA. We currently have available to
us TGFβ3 and Ap-1 regulated promoters that represent non-classical EREs and we are in the process of testing the effect of Smad3 and ER on the activity of these plasmids.

Although Smad3 does not affect ER transcription in Cos1 cells, the question still remains whether ER could affect Smad3 transcriptional activity. Recently, we obtained the Smad3 responsive p3TP-Lux luciferase reporter plasmid from Dr. Massague (Rockefeller University) which contains the TGFβ responsive element of the plasminogen activator inhibitor-1 (PAI-1) downstream of three TPA-responsive elements of the human collagenase gene\textsuperscript{16}. This plasmid has been well characterized as a TGFβ responsive promoter and overexpression of Smad3 by transient transfection increases its activity\textsuperscript{16, 17, 18, 19}. We have transfected this plasmid into Cos1 cells along with Smad3, ER and pCH110 as a transfection efficiency control in the presence or absence of 10nM estradiol. Results indicate that ERα (p<0.001) and ERβ\textsubscript{1} (p<0.05) inhibit p3TP-Lux transcription in the presence of estradiol. To assess the specificity of estrogen effects on Smad3 transcriptional activity, we utilized the anti-estrogens tamoxifen and ICI 182,780. The inhibitory effect of ERα and ERβ\textsubscript{1} in the presence of estradiol on p3TP-lux was reversed by both 4OH-tamoxifen (100nM) and ICI 182,780 (100nM) suggesting that the effect of ER on Smad3 transcription is ligand dependent. ERβ\textsubscript{2} and ERβ\textsubscript{3} did not affect Smad3 transcriptional activity. However, upon western blot analysis of our transfected cells, it appears as though ERβ\textsubscript{2} and ERβ\textsubscript{3} are expressed at a much lower level compared to ERβ\textsubscript{1} and ERα which may account for the differences. Although this is unlikely since the variant receptors are not ligand activated, we are currently attempting to increase the protein expression level of these variants in our Cos1 cells to determine whether this higher level of expression affects Smad3 transcriptional activity on p3TP-Lux.

4. KEY RESEARCH ACCOMPLISHMENTS

\begin{itemize}
\item Cloning of ERα, ERβ\textsubscript{1}, ERβ\textsubscript{2}, ERβ\textsubscript{3} and Smad3 into appropriate vectors.
\item Co-immunoprecipitation assays \textit{in vitro} and in Cos1 cells completed.
\item Optimization of ERE-luc assays in Cos1 cells.
\item Smad3 does not affect ER transcriptional activity on a vitellogenin ERE regulated promoter in Cos1 cells.
\item Optimization and validation of the Smad3 reporter gene, p3TP-Lux.
\item ERα and ERβ\textsubscript{1} inhibit Smad3 transcription on p3TP-Lux in a ligand-dependent manner and this effect is prevented in the presence of the anti-estrogens 4OH-tamoxifen and ICI 182,780.
\item ERβ\textsubscript{2} and ERβ\textsubscript{3} do not appear to affect Smad3 transcription on the p3TP-lux reporter.
\end{itemize}
5. REPORTABLE OUTCOMES


6. CONCLUSIONS

Our preliminary experiments together with our current results suggest that a cross-talk between the ER and TGFβ signalling pathways exists. Our preliminary data suggested that an interaction between Smad3 and ER exists in the yeast two-hybrid system. Our current data suggests that a weak physical interaction between Smad3 and ERα or ERβ1 exists in vitro, although we have not been able to detect this in vivo in Cos1 cells. Furthermore, increased Smad3 expression does not effect ER transcriptional activity as measured through activation of an ERE luciferase reporter gene. However, ER is able to repress Smad3 transcriptional activity on the Smad reporter gene p3TP-lux in a ligand dependent manner. The mechanisms by which this occurs are currently being investigated. Overall the data support the hypothesis that ER interacts with the TGFβ signalling pathway.

7. REFERENCES

7. Simon et al. Unpublished data.
Appendix 1
CROSS-TALK BETWEEN THE TGF-β AND ER SIGNALING PATHWAYS.
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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.