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Introduction:

The overall theme for this study focuses on the role of TGF-β signaling in normal prostate development. TGF-β growth factors bind to a heteromeric surface receptor (TβIR/TβIIR). This binding results in a cascade of intracellular signaling mediated by the Smad family of proteins with Smad2,3 and 4 up-regulating the signaling and Smad6,7 inhibiting the signaling. The mechanisms of signal transduction by growth factors represents an important area of investigation to understand fetal development and tissue differentiation. Resumption of patterns of gene expression that are typically embryonic has been associated with tumorigenic conversion of cells. Our studies are focused on the transduction of signal by transforming growth factor-β (TGF-β) during development. Smad 2 and Smad 3 are activated following ligand binding to specific receptors for TGF-β and the molecules must be phosphorylated to be active in changing gene transcription. Smad 6 and 7 inhibit the activation of the TGF-β signaling pathway to diminish the growth factor related response. The on-going studies are characterizing patterns of Smad gene expression during prostate gland development to examine the effects on cell growth and differentiation. The studies are focused on the molecular mechanism of TGF-β signal transduction and the role of these mechanisms in morphogenesis.

Body:

Specific Aim 1. To define the developmental and temporo-spatial expression of Smad genes during embryonic prostate development (i) in vivo and (ii) in neonatal mouse prostate organ culture.

The first specific aim is foundational for the project and contains three subaims; 1a) to develop reagents and protocols; 1b) to analyze mRNA; 1c) to analyze protein. In Specific Aim 1 the baseline analyses were completed to justify the subsequent aims and the descriptive data represented the basis for controlling the mechanistic studies in Aims 2 and 3. The results of the first specific aim became critical to the premise for this entire study and as reported in the Preliminary Results for the proposal there was a firm basis for studying the TGF-β signaling pathway during organogenesis. The preliminary data reported that, "Cultured mouse anterior prostates were harvested for RNA extraction, reverse transcription and PCR amplification. Transcripts for TGF-β1,2,3, TβIR, TβIIR, Smad2,3,4,6,7 were found to be expressed during prostate growth in organ culture. Both androgen receptor and probasin were also expressed at mRNA level in cultured prostates." Continued investigation between the time of submission to funding of the proposal resulted in development of the reagents and protocols used in subsequent studies thus completing the originally proposed reagents and protocols in 1a, however the nature of these investigations has required continued improvements of the model system to optimize the outcomes. Additional data regarding the temporal and spatial expression of the different members of the TGF-β signaling pathway was completed to serve as the comparison of outcomes for interventional analyses in Aims 2 and 3, (1b,1c). These results were very descriptive and related to the histomorphology of the tissues and further provided a basis for controls for subsequent studies in Specific Aims 2 & 3. Thus analysis of Smad mRNA (1b) and protein (1c) in normal and experimentally manipulated tissues is a continuous process that allows the normalization of results and comparisons. Rather than serving as an endpoint, these descriptive studies are the foundation for the molecularly based investigations that examine specific mechanisms.

Outcomes from studies that were reported in our October 14, 2004 Progress Report demonstrate that while the emphasis was on the Smad2 signaling pathway that the analysis of the

distribution and modification of the Smad molecules in the tissue was a critical event related to organogenesis and represented continued analysis of the Specific Aim 1 goals as foundational to the completion of the goals for the other aims (1b, 1c). "Smad 2 expression and post-translational activation by phosphorylation is critical for the intracellular propagation of the TGF-β signal. The earliest periods of organ development are marked by a dramatic increase in Smad 2. This Smad 2 is phosphorylated indicating that it has responded to the binding of the growth factor ligand to the signaling receptor. At 7 days the level of all of the proliferating prostatic cells have high levels of Smad 2, thus TGF-β signaling at this stage is directly linked to cell proliferation and growth in organ size. Of interest is that all three TGF-β receptors are present on these cells, both the Type I and Type II receptors that are required to transmit the signal and the Type III receptor that is non-signaling. The presence of the TGF-β Type III receptor has not been previously demonstrated in the developing prostate and has never been demonstrated in prostate cancer. These observations potentially demonstrate fundamental differences in the TGF-β signaling events that occur during development and further examination of the growth factor receptors and their link to gland development are planned."

In the first progress report of 10-15-2002 it was indicated that continued development of new reagents and protocols was underway to more appropriately address the research questions (1a). "The use of a genetically engineered strain of mice that over-express Smad 2 in epithelial cells. This over-expression represents an important opportunity to further characterize the role of Smad2 in the TGF-β3 signaling pathway by breeding to a TGF-β3 null mutant mouse strain. This model represents a unique opportunity to characterize the Smad2 mechanism since increased levels of Smad2 gene expression may overcome the loss of function of the TGF-β3 ligand. These genetically engineered tissues can be examined with respect to the effects of TGF-β1 and TGF-β2 on modification of the Smad2 and the consequent activation of TGF-β-type specific gene expression. At this time the lines have been crossed and several generations of mice generated. The process of defining genetic homogeneity is underway and in the next funding year these mice will be available to conduct unique opportunities not available by any other mechanism. The potential to develop a genetic rescue of the null mutant phenotype will provide novel insight into the mechanism overall and provide a means to determine whether specific types of TGF-β related interventions could be used to alter prostate growth and differentiation". Other examples of continued development of reagents and protocols are included in the discussion of progress on Specific Aims 2 and 3.

Continuing Analysis during No-Cost Extension Year related to Specific Aim 1:

A primary outcome measure in all the studies is the analysis of mRNA (1b) and proteins (1c) from the genes involved in the TGF-β signaling pathway. Thus it will continue to be necessary to analyze these molecules in both the controls and the experimental samples. The comparison of results between controls and experimentals is essential for analyzing the impact of interventions to disrupt the TGF-β/Smad signaling. Although the reagents and protocols originally proposed have been completed and are in use continued development of new approaches is essential to the progress of these projects (1a). The development of appropriate model systems to analyze the TGF-β/Smad pathway is an on-going activity as new genetic approaches and gene expression modification approaches are developed. In both Aims 2 & 3 continued optimization of reagents and protocols, such as Cre-lox, siRNA, and overexpression models, will be occurring to generate the most reproducible results (1a).

Status of Specific Aim 1.

1a: Original reagents and protocols developed and in use, new approaches continuing to be developed to allow the optimal analysis of the mechanisms.

1b: Initial characterization of baseline descriptive distribution of mRNA is completed and these studies continue as the basis for establishing the controls in each experiment to determine the impact of specific interventions.

1c: Initial characterization of the baseline descriptive distribution of protein is completed and these analyses continue as outcome measures for control and experimental groups.

Publication:

The results of the analyses described in Specific Aim 1 will be incorporated into the publications that are in preparation reporting the results of analysis of the TGF-β signaling pathway during organogenesis.

XM Cui, CF Shuler TGF-β Type III Receptor specific for prostate cell proliferation at initiation of organogenesis. (manuscript in preparation)

Specific Aim 2. To determine the molecular mechanisms of TGF-β pathway restricted Smad2 and Smad3 in regulating prostate ductal branching morphogenesis and cytodifferentiation in serumless organ culture.

The second specific aim is mechanistic and based on the descriptive foundation findings in SA1 and contains three subaims; 2a) effects of inhibiting gene expression; 2b) organ-specific effect of gene expression; 2c) effects of gene over expression. The binding of TGF-β to the TGF-β receptors results in the activation of a kinase activity in the receptors and the phosphorylation of Smad 2/3, which initiates the positive signaling of the TGF-β pathway. Thus the results of this aim analyze the outcomes in cells and tissues of TGF-β ligand binding to the specific receptors.

One approach to determine the importance of a molecule in the events required for organogenesis is to selectively eliminate that molecule from the cell and observe the effects (2a). Since the Smad molecules are intracellular there is a requirement to accomplish their removal through methods that interrupt either the generation of specific mRNA or translation of the specific mRNA. In the current period of study we have been optimizing techniques to eliminate the signaling mediated by Smad 2 through methods using siRNA (short interfering RNA). These molecules are introduced into the cells in culture and then bind to the Smad 2 mRNA and prevent translation of the protein. We have been successful in introducting the Smad 2-specific siRNA into cells in organ culture. Subsequent analysis of the cells has shown that the siRNA greatly reduced the amount of Smad 2 protein without altering the amount of Smad 2 gene expression. The optimal concentrations of siRNA have been determined that can be used in organ culture without affecting the normal progression of development. This technology can be utilized to specifically analyze the effects of loss of just one protein from the signaling pathway and determine the impact on organogenesis. The siRNA approach is a loss-of-function strategy that provides a way to examine deficiencies of specific molecules. The siRNA strategy is a change from the originally proposed antisense oligonucleotide approach to inhibit the genes that was based on the observation of some generalized chemical toxicity of the oligos. The siRNA approach is a new strategy that effectively knocks-down gene expression without the associated

toxicity. The Dharmacon Company has developed siRNA reagents with appropriate controls for all the molecules in the TGF-β signaling pathway allowing the experimentation to be carefully planned and controlled. Further studies are on-going to use the siRNA approach to alter the TGF-β signaling and determine the impacts on cell proliferation and cell death.

The expression and activation of Smad 2 during normal development in the post-natal period continues to be a productive area of investigation (2b). Smad 2 expression and post-translational activation by phosphorylation is critical for the intracellular propagation of the TGF-β signal. The earliest periods of organ development are marked by a dramatic increase in Smad 2. This Smad 2 is phosphorylated indicating that it has responded to the binding of the growth factor ligand to the signaling receptor. At 7 days the level of all of the proliferating prostatic cells have high levels of Smad 2, thus TGF-β signaling at this stage is directly linked to cell proliferation and growth in organ size. Of interest is that all three TGF-β receptors are present on these cells, both the Type I and Type II receptors that are required to transmit the signal and the Type III receptor that is non-signaling. The presence of the TGF-β Type III receptor has not been previously demonstrated in the developing prostate and has never been demonstrated in prostate cancer. These observations potentially demonstrate fundamental differences in the TGF-β signaling events that occur during development and further examination of the growth factor receptors and their link to gland development are planned.

The effects of excessive expression of Smad 2 have been analyzed in a genetic model developed to address some deficiencies of the originally proposed adenovirus approach.(2c) Originally it was proposed to use an adenovirus model to transfer new genetic elements into the cell however it was found that there was sufficient delay in the expression of the DNA and presence of the new protein that critical developmental events were completed before the new gene had an effect. Consequently an alternative genetic based strategy was used and reported in the October 14, 2004 Progress report. "A transgenic animal model has been developed that permits overexpression of Smad 2 in epithelial cells. An epithelial cell specific promoter, K14, has been linked to the Smad 2 gene and transgenic mice generated. This DNA promoter-gene construct has been shown to greatly increase Smad-2 expression in cells in transgenic animals. We are utilizing this model system to advantage to analyze the effects of Smad-2 expression on the development of the prostate. If Smad 2 is directly linked to cell proliferation then increased levels of Smad 2 should be linked to enlargement of the organ and replicate events that are observed in benign prostatic hyperplasia and in prostatic carcinoma. This transgenic mouse model is a unique approach to examine gain-of-function outcomes on the developing tissue. The future studies are planned to analyze the impact of excessive Smad 2 on prostate development."

Another strategy that is being develop to analyze tissue-specific expression patterns linked to TGF-β signaling is through developing additional models (1a) and applying them to all three sub-aims (2a, 2b, 2c). A TGF-β promoter cloned into a DNA construct that contains the sequences for two genes, GFP (green fluorescent protein) and Cre (Cre recombinase). This animal model will permit the patterns of expression of TGF-β to be analyzed in specific tissues through the GFP and allow tissues specific elimination of genes by Cre. The DNA constructs have been produced and injected into fertilized oocytes. These oocytes have been used to generate lines of mice with this unique pattern of gene expression. Breeding of the mice is continuing to generate sufficient numbers of animals to determine if the transgene expression goals have been met. Once this has been ascertained these animals will result in a unique model to specifically characterize the role of defined genes during organogenesis. We have secured through a collaboration with Dr. Michael Weinstein at Ohio State University a strain of mice that

have the Smad2 gene flanked by loxP sites so that the gene can be selectively eliminated in cells expressing Cre. This model will allow very precise alterations in gene expression by either knocking out or activating genes in precise groups of cells.

Continuing Analysis during No-Cost Extension Year related to Specific Aim 2:

The studies in Specific Aim 2 have been very productive and generated interesting results. These studies in all three subaims (2a, 2b, 2c) continue. The results that have been obtained are being replicated to determine the reproducibility of the results. Analysis of sufficient replicates will generate sufficient data for publication of the findings. The approaches that will continue in the No-Cost Extension Year are as previously described.

Status of Specific Aim 2.

2a: The inhibition of Smad2 has been accomplished using siRNA technology and the reduction in the protein documented. Replication of the experiments are continuing to generate sufficient data for publication.

2b: The TGF-β Type III receptor has been specifically localized to proliferating neonatal prostate cells and the function of this receptor in development is being characterized. The phosphorylation of Smad2 has been linked to increased levels of cell proliferation at the earliest stages of organogenesis. The results are being analyzed and replicated to be used in publications of the findings.

2c :An epithelial-specific promoter driving Smad2 gene expression has been introduced into a transgenic mouse model to permit gain-of-function studies that could not be satisfactorily achieved with the adenovirus approach. The effects of enhanced expressed are being analyzed and replicated.

Publication:

The analysis and replication of the results from the studies in Specific Aim 2 is on-going. When those results have been sufficiently analyzed for reproducibly they will be incorporated into publications. It is anticipated that these publications will be generated during the No-Cost Extension period.

Specific Aim 3. To define the biological function of the feedback inhibitory Smad7 and Smad6 proteins during mouse prostate growth and development in organ culture.

The third specific aim is mechanistic and also based on the descriptive foundation findings in SA1. This aim contains three subaims; 3a) organ-specific effect of gene expression; 3b) effects of inhibiting gene expression; 3c) effects of gene over expression. The induction of the intracellular signaling pathway for TGF-β can be inhibited by Smad6/7. The induction of these inhibitory Smads can be linked to regulation of the propagation of the TGF-β signaling and thus Smad6/7 may be thought of as antagonistic to Smad 2/3, which were the focus of Specific Aim 2. Thus the results of this aim analyze the outcomes in cells and tissues when the intracellular pathway activated by TGF-β ligand binding is inhibited.

The initial characterization of the baseline Smad6/7 expression during organogenesis was included in the foundational studies in Specific Aim 1 (3a). Smad6/7 remain outcome markers that are used in both Specific Aim 2 and Specific Aim 3 to establish the basis for interpretation in experimental tissue with modulated gene expression. The studies in 3b are being initiated and

consequently the characterizations in 3a will occupy additional effort and the results will have important meaning to the interpretation of the interventions to alter gene expression.

The use of antisense oligonucleotides was originally proposed to inhibit the Smad6 and Smad7 in the cultured tissues (3b). It was found that antisense oligonucleotides have considerable toxicity as a result of the chemistry of these molecules and that that toxicity can confound the results. A new technology, siRNA. has been developed that accomplishes the same outcome as antisense oligonucleotides, to knock-down the level of gene expression. We have chosen to shift to the siRNA technology to knock-down the level of gene expression. It is to our considerable advantage that the Dharmacon Corporation has developed a full set of siRNA for the TGF-β signaling pathway molecules including Smad2,3,4,6 & 7. Thus we are now using this more effective technology to evaluate the effects of knocking down gene expression. As reported in the October 14, 2004 Progress Report we have optimized the siRNA technology for use in the organ culture model system. "One approach to determine the importance of a molecule in the events required for organogenesis is to selectively eliminate that molecule from the cell and observe the effects. Since the Smad molecules are intracellular there is a requirement to accomplish their removal through methods that interrupt either the generation of specific mRNA or translation of the specific mRNA. In the current period of study we have been optimizing techniques to eliminate the signaling through methods using siRNA (short interfering RNA)." We now will proceed to use this optimized technology to analyze the Smad6 and Smad7 effects in organ culture.

A complication was found in evaluating the use of the adenovirus system in the organ cultures that was related to the timing of gene expression from DNA transferred to cells by the adenovirus (3c). It was found that the infection of the cell by the virus occurred readily however the movement of the new DNA elements to the nucleus and subsequent expression of the new genes took a period of time. In the rapidly developing organs the availability of new protein from the DNA transferred by the adenovirus often was not present at sufficient levels at the correct period of time to be effective. This was the same problem observed working on studies in Specific Aim 2. In Specific Aim 2 we were able to develop a keratin gene promoter driving Smad2 to achieve an alternative means of over-expression to enable to accomplish our aims. Alternative approaches for Smad6 and Smad7 over-expression are being investigated. When those approaches are developed it will be possible to assess the impact of Smad 6/7 overexpression on organogenesis.

Continuing Analysis during No-Cost Extension Year related to Specific Aim 3:

The studies in Specific Aim 3 have had variable levels of activity. The analysis of Smad6/7 during organogenesis is a component of the characterizations of the tissues in control and experimental samples (3a). The inhibition of Smad 6/7 using siRNA is beginning using methodologies that have been optimized in the laboratory (3b). The protocols to achieve over expression of Smad6/7 have been complicated by technical difficulties with the adenovirus approach (3c) and have required investigation of new genetic models. Additional replicates and data analysis will continue in the No-Cost Extension Year are as previously described.

Status of Specific Aim 3.

3a: The localization of Smad6/7 in the tissues was accomplished as part of SA1. Further analysis of the role of these inhibitory Smads is coordinated with the other experiments using the

SA1 findings as outcome markers for control and experimental tissues. The Smad6/7 patterns will be compared with the other molecules in the TGF-β signaling pathway.

3b: The inhibition of Smad6/7 by siRNA is being initiated. The siRNA technology has been established in the laboratory and the reagents are available from Dharmacon company. The impact of inhibition of Smad6/7 will be analyzed during organogenesis.

3c: The adenoviral approach to over-express the genes had a technical problem such that expression did not occur with the appropriate timing. Genetic models for over expression of Smad6/7 are being developed to achieve this sub-aim.

Publication:

The analysis and replication of the results from the studies in Specific Aim 3 has been initiated. The generation of sufficient reproducible data will allow for the generation of a publications during the No-Cost Extension period.

Conclusion:

These studies are focused on the specific signal transduction mechanism related to TGF-β binding to specific receptors, subsequent activation of kinase activity and activation of downstream mediators through phosphorylation. The development of the technologies to evaluate loss-of-function and gain-of-function of this signaling pathway will allow focus of specific cell differentiation under the control of TGF-β signaling. The use of novel transgenic mice provides approaches that are focused on specific elements of the signaling pathway. Further investigation of the fundamental mechanisms will provide improved characterization of control of the critical developmental events.

Appendices:

None