PJN
ISSN 1680-5194

PAKISTAN JOURNAL OF NUTRITION

ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorpjn@gmail.com

Pakistan Journal of Nutrition 1(1) : 53-55, 2002

Expression of Bovine Mammary Gland SMAD 4 and its Relationship to BRCA1

[1]Yongji Chung and R.C.Gorewit
[1]Department of Pathology, Northwestern University, Evanston Hospital Evanston, IL 60201
Lactation Physiology Laboratory, Department of Animal Science, Cornell University,
Ithaca, New York 14853

Abstract: Little is known about the role of TGF-ß in ruminant mammary growth and development. The literature suggests that TGF-ß could play an active role in influencing early mammary development and involution. SMADs are proteins that function as intracellular signaling effectors for the TGF-ß super family of polypeptides. We cloned bovine SMAD 4 and examined its mRNA expression in the mammary gland at various developmental stages. Sequencing analysis showed that bovine SMAD 4 had 96 and 94% homology to human and rat SMAD 4, respectively. Therefore, SMAD 4 appeared to be a very conserved gene in these species. SMAD 4 mRNA expression was highest in early pregnancy and involution, and then decreased in late pregnancy and maintained a low level through lactation. We speculate that SMAD 4 is involved in induction of regulatory genes involved in mammary gland growth and apoptosis. BRCA1 is a breast and ovarian cancer-specific tumor suppressor protein, with properties of a transcription factor involved in cell cycle regulation, DNA repair and apoptosis. BRCA1 gene expression is induced by steroid hormones and its pattern of mRNA expression mimicks that for TGF-ß and SMAD 4. We thought it of interest to determine if various doses of TGF- ß would induce BRCA1 gene expression. TGF- $ß_1$ treated bovine mammary cells showed a dose dependent increase in BRCA1 mRNA expression compared to control treatments. Our results suggest that TGF-ß1, SMAD 4 and BRCA1 may play important roles in regulating ruminant mammary cell proliferation, differentiation, and involution or apoptosis.

Key words: SMAD 4, BRCA1, mammary, development

Introduction

TGF-ß regulates ductal growth and morphogenesis in the mouse mammary gland (Daniel *et al.*, 1989; Silberstein and Daniel, 1987). The growth factor is a potent inhibitor of mammary development prior to puberty, but does not influence mammary development during pregnancy in mice (Daniel *et al.*, 1989; Robinson *et al.*, 1991). In cattle, very little is known about the role of TGF- ß in mammary growth and development.

Plaut, 1993 examined receptor binding of TGF-ß1 to bovine mammary membranes from pubertal and lactating animals. Specific binding of TGF-ß1 was higher during the prepubertal and pubertal periods than during lactation, suggesting that the growth factor could play an active role in influencing early mammary development. Plath *et al.*, 1997 showed expression of TGF- ß1 during mammogenesis, lactogenesis, galactopoiesis and involution in bovine mammary gland. TGF- ß1 expression was higher during mammogenesis of virgin heifers and involution than during lactogenesis and galactopiesis.

Woodward *et al.*, 1993 characterized TGF- ß receptors and their autoregulation, and the growth response to TGF- ß1 and TGF- ß2 in cultured bovine mammary epithelium (MAC-T) and fibroblasts. Affinity labelling studies revealed that fibroblast and epithelial cells contained type I, II, and III (betaglycan) receptors, with the type III receptor being the predominant binding component. Pre-incubation of MAC-T cells with 50 pM TGF- ß1 or TGF- ß2 markedly downregulated TGF-beta receptors.

Proliferative response was measured using both total DNA and 3H-thymidine incorporation. Both TGF-beta isoforms inhibited MAC-T and fibroblast proliferation. Inhibition was reversible as shown by return of cellular proliferation to control levels following TGF-beta removal. Although growth inhibition was not transient as culture of MAC-T cells in TGF-beta resulted in sustained inhibition of proliferation for at least 144 h. Although it appears that TGF-ß is involved in the regulation of mammary growth and development, the mechanism(s) of its action is unknown.

SMADs are a class of proteins that function as intracellular signaling effectors for the TGF-ß superfamily of secreted polypeptides. SMADs received their name as a contraction of the names of the *C. elegans* Sma and Drosophila Mad, the first identified members of this class of signaling effectors. In all vertebrate cells studied, TGF- ß signals through sequential activation of two cell surface receptor serine-threonine kinases, that phosphorylate SMAD 2 and/or SMAD 3 (Zhang *et al.*, 1996; Zhang-Ying *et al.*, 1998). Phosphorylated SMAD 2 or SMAD 3, together with SMAD 4, translocate into the nucleus and associate with other transcription factors, leading to the activation of specific gene transcription (Derynck-Rik *et al.*, 1998; Heldin *et al.*, 1997; Labbe *et al.*, 1998; Lagna *et al.*, 1996; Liu *et al.*, 1997).

BRCA1 is a breast and ovarian cancer-specific tumor suppressor protein, with properties of a transcription factor involved in cell cycle regulation, DNA repair and apoptosis (Chapman and Verma, 1996; Paterson, 1998). BRCA1 inhibits proliferation of breast cancer cells (Paterson, 1998). We have recently shown that BRCA1 is differentially expressed in normal bovine mammary tissue. Moreover, the profile of expression is similar to that of TGFb1 expression . BRCA1 mRNA expression was examined over developmental stages from biopsied mammary tissue by RNase protection assay . The mammary tissues of early pregnancy heifers (3 months) showed much higher mammary BRCA1 mRNA expression than other mammary developmental stages. BRCA1 expression declined during the remainder of pregnancy and remained low for lactating cows. However, its expression increased when involution proceeded.

Since it has been shown in the literature that TGF-B1 is differentially expressed at various stages of mammary development, lactation and involution, we thought it of interest to determine if bovine mammary gland SMAD4, the intracellular signaling effector for the TGF-ß superfamily was also differentially expressed in the same manner. In an additional experiment, we determined if TGFb1 was capable of inducing transcription of BRCA1 in cultured bovine mammary cells..

Materials and Methods

Cloning SMAD4 : Total RNA was isolated from 0.5g of mammary tissue of non-lactating cows obtained from the slaughterhouse. Tissue was homogenized by a Polytron in Tri reagent (Molecular Research Center) and the manufacturer's recommendations were followed with regard to homogenization of tissue. Total RNA (1μg) was used for reverse transcription (RT) with a cycle kit (Invitrogen). RT procedures were according to manufacturer's recommendations and 2/10 of RT product (4μl) was used for PCR amplification. The PCR steps were performed as follows: 95 °C for 3 min, then 30 cycles of amplification (95 °C × 30 sec, 55 °C

```
bSMAD4  (1)     tgtgaatccatatcactacgaacgagttgtgtcacctggaattgatctct
hSMAD4  (509)   tgtgaatccatatacactacgaacgagttgtatcacctggaattgatctct
rSMAD4  (509)   tgtgaacccatatcactatgagcgggttgtctcacctggaattgatctct
bSMAD4  (51)    caggattaacactgcagagtaatgctccaccaagtatgttggtgaaggat
hSMAD4  (559)   caggattaacactgcagagtaatgctccatcaagtatgatggtgaaggat
rSMAD4  (559)   ctggattaacactgcagagtaatgctccaccaagtatgttagtgaaggat
bSMAD4  (101)   gaatatgttcatg
hSMAD4  (609)   gaatatgtgcatg
rSMAD4  (609)   gaatatgttcatg

Bovine  SMAD4   1-113    (113 bps)   Homology
Human   SMAD4   509-621  (113bps)    96 %
Rat     SMAD4   509-621  (113 bps)   94%
```

Fig. 1: Partial sequence of bovine SMAD4 cDNA and sequence comparisons with human and rat (Identical bases are underlined). Percent homologies between species are also shown.

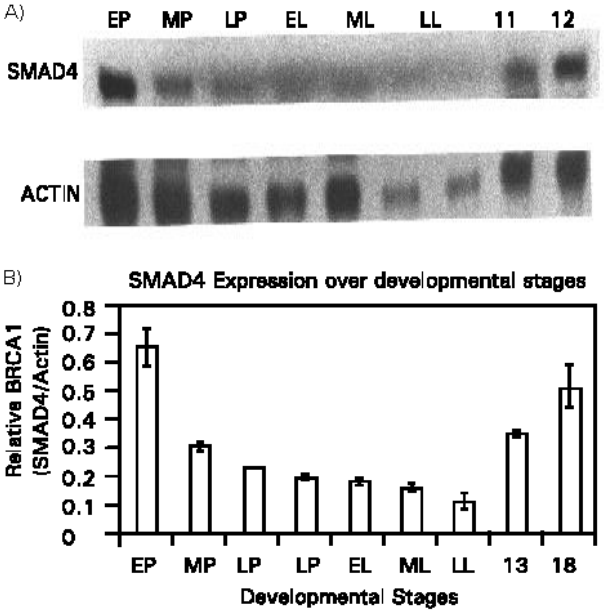

Fig. 2: Bovine mammary gland SMAD4 expression over various developmental stages. (A) RNase protection assay (RPA) was used to quantify bovine SMAD4 mRNA expression. Each lane contains 15μg of total RNA extracted from the bovine mammary gland. (B) Quantification of Relative SMAD4 mRNA expression to ß-actin mRNA expression is shown. EP (Early Pregnancy): 3 months; MP (Mid Pregnancy): 5 months; LP (Late Pregnancy): 6 months; LP (Late Pregnancy): 8 months; EL (Early Lactation): 3 months; ML (Mid Lactation): 6 months; LL (Late Lactation): 9 months; 6wk Involution, and 8wk Involution. Representative radiographs are shown in A. Means and standard errors are shown for three separate electrophoretic runs of three animals at each developmental stage in B.

×60 sec, 72 °C × 1 min), ending with 72 °C ×3 min. Taq polymerase (Gibco) was used for amplification. The primer set used for SMAD4 was 5′ gtgtgaatccatatcactac 3′/ 5′ aacgatggctgtccctcaaa 3′. The amplified product (15 kb) was cloned using the TA-Cloning Kit (Invitrogen). The procedures used were according to the manufacture's instructions. The cloned element was sequenced at the Cornell University sequencing facility.

Differential expression of bovine mammary SMAD4 :

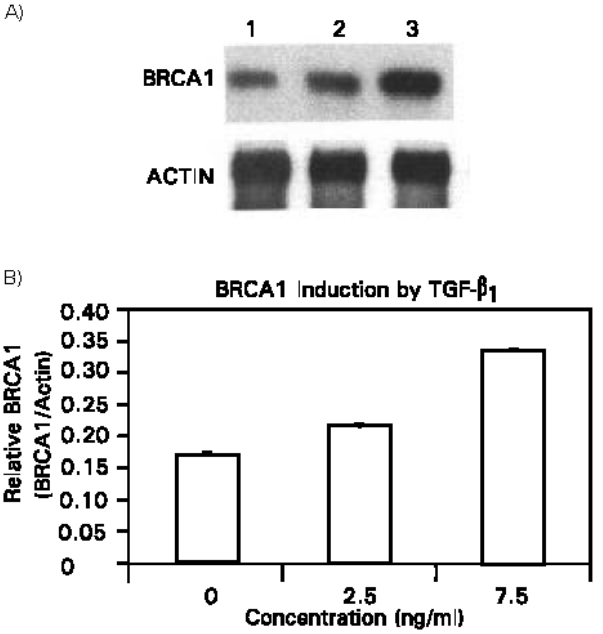

Fig. 3: BRCA1 induction by TGF-ß$_1$ in BME cells. (A) RNase protection assay for analysis of bovine BRCA1 mRNA induction within 1 hr by TGF-ß$_1$. Lane 1: Control, Lane 2: 3.76 ng/ml of TGF-ß$_1$, Lane 3: 14.3 ng/ml of TGF-ß$_1$) Lanes 1-3 contain 15 μg of total RNA isolated from cultured BME cells. (B) Quantification of BRCA1 mRNA expression showed in (A). Relative BRCA1 mRNA expression to ß-actin mRNA expression is shown.

Mammary Biopsy: Mammary tissue was obtained by biopsy from conscious female Holstein cattle in the following physiological stages (primiparous and three and eight months pregnant; multiparous and lactating for 3-5 months (early lactation); multiparous and lactating for 6-8 months (mid lactation); multiparous and lactating for 9-11 months (late lactation); and multiparous and undergoing mammary involution (three, six, and eight weeks). Three animals were biopsied per each developmental stage.

Animals were brought into a surgery suite and the point of tissue excision was anesthetized with lidocane. Five ml lidocane was also injected around the excision site. Approximately 10 grams of tissue was removed containing parenchymal elements. Blood vessels were cauterized or sutured to prevent further bleeding after biopsy removal. The excision site was closed with self-dissolving suture. Animals were given an injection of penicillin and returned to the Cornell University dairy farm.

Ribonuclease Protection Assay (RPA): Total RNA was extracted using Tri reagent (Molecular Research Center), as decsribed by the supplier. The SMAD4 Primer set was 5′ gtgtgaatccatatcactac 3′/ 5′atttaggtgacactatagaaaacgatggctgtccctcaaa3′. The reverse primer contained the SP6 RNA polymerase binding site (underlined) to make antisense ribo-probe. Approximate 150 nt biotin labeled SMAD4 antisense ribo-probes were synthesized by MAXI Script (Ambion) according to the manufacturer's recommendations. The synthesized probes were gel purified and hybridized with 20 μg of total RNA to protect a fragment of the expected length. After nuclease digestion, the protected fragment was eletrophoresed, transferred to nylon membrane by electro-transfer, and detected by Ambion's Bright Star Bio-Detection Kit SMAD4 mRNA was quantified by Photo Image IS1000 with arbitrary units corrected for the expression of the control, actin mRNA (SMAD4 /Actin).

BRCA1 induction by TGF- ß: In order to determine the effect of TGF- ß on the induction of BRCA1 expression BME cells were treated with TGF- $ß_1$ and RPA was performed. Cells ($2x10^6$) were plated in 100 mm dishes and grown to confluency. Before hormone treatment, cells were serum starved for 24h and media was changed without serum twice. Cells were then treated with various concentrations of TGF- $ß_1$ (0, 3.76, 14.3 ng/ml). After 2h, medium was discarded and cells were lysed by Tri reagent LS (Molecular Research Center) to isolate total RNA. Fifteen microgram of isolated total RNA was used for the RNase protection assay, as described above.

Results and Discussion

Cloning of SMAD4 : We cloned a partial cDNA of bovine SMAD4 by RT-PCR and confirmed it by DNA sequencing (Fig. 1). Sequencing analysis showed that it has 96 % homology to the human and 94 % to the rat SMAD4. Therefore, SMAD4 appeared to be a very conserved gene in these species. All PCR primers used for molecular cloning of SMAD4 were based on human sequences.

Differential expression of bovine mammary SMAD4 : SMAD4 mRNA expression was high in early pregnancy and involution, and then decreased in late pregnancy and maintained a low level through lactation (Fig. 2). There is a great degree of cell proliferation during early pregnancy and massive tissue remodeling occurring in the mammary gland during involution. Therefore, we speculate that SMAD4 is involved in induction of growth and apoptosis regulatory genes in these developmental stages with SMAD2, or 3.

It is not known exactly what factors are involved in regulation of SMAD4 gene induction. In general, TGF- ß binding to its receptor induces phosphorylation of SMAD2, 3 and phosphorylated SMAD2, 3 recruit SMAD4 to generate a complex (Derynck-Rik, 1998). However, there is no report that TGF-ß induces up-regulation of SMAD4 in the mammary gland. Our data suggests that SMAD 4 is constitutively expressed over satges of mammary development and involution.

BRCA1 induction by TGF- ß: To address if TGF-ß can induce BRCA1 gene induction *in vitro*, we treated BME cells with TGF- $ß_1$ and then performed RPA to observe BRCA1 induction (Fig. 3). TGF- $ß_1$ treated cells showed a dose dependent increases of BRCA1 induction compared to control treatments.

Transcription factors that cooperate with SMAD proteins, to regulate transcription of certain genes, include FAST-1, a winged-helix transcription factor, which mediates activin induction of the Mix.2 gene during embryonic frog development , c-Jun, c-Fos, ATF2, and vitamin D receptor, which interact with phosphorylated SMAD3 to mediate TGF- ß-induced transcription of various genes (Zhang-Ying, 1998).

Zawel *et al.*, 1998 identified a palindromic SMAD binding element. GTCTAGAC, by selecting for SMAD3 and SMADd4 binding sequences from a pool of random oligonucleotides. The three dimensional structure of the SMAD3 MH1 domain (N domain) indicates that an MH1 monomer binds precisely to a 4-bp sequence, -AGAC- (Labbe *et al.*, 1998; Zhang-Ying *et al.*, 1998). This AGAC SMAD binding element (SBE) should appear on average once every 256 bp in the genome ($1:4^4$). Thus, most genes that contain binding sites for SMAD partner transcription factor, such as FAST-1, AP-1, and transcription factor uE3 (TFE3) , will have SBEs in their promoters. Not all genes with binding sites for such transcription factors are transcriptionally responsive to TGF- ß. Therefore, it is unclear what controls the specificity of TGF-ß signaling at the transcription level.

The human BRCA1 promoter has several copies of -AGAC-element. However, we have not seen either palindromic repeats or direct tandem repeats of AGAC, used as a response elements for SMAD3/4 complex binding, in BRCA1 promoter sequences. Although this AGAC single repeat would expect to show weak binding to SMAD3/4, we can not neglect the possibility of these AGAC single repeats for BRCA1 gene induction. Needless to say, more studies are necessary to investigate the TGF-ß effect on regulation of BRCA1 gene induction.

Our studies are the first to describe gene expression for SMAD 4 through various stages of mammary gland development and involution. Expression patterns in the bovine were identical to those for TGF-B and BRCA1 during the same physiological stages. TGF-B1 induced expression of BRCA1 in cultured bovine mammary cells, thus, suggesting that TGF-B and its SMAD signaling system may be involved in the regulation of BRCA1 gene expression. Further studies are necessary to define the synergistic role of these factors in normal and neoplastic mammary gland growth, differentiation, and involution.

References

Chapman, M.S. and I. M. Verma, 1996. Transcriptional activation by BRCA1.Nature, 382: 678-679.

Daniel, C.W., G.B. Silberstein, K.Van Horn, P.Strickland and S. Robinson, 1989. TGF-β1- induced inhibition of mouse mammary ductal growth: developmental specificity and characterization. Dev. Biol., 135:20.

Derynck-Rik. Zhang-Ying. Feng-Xin-Hua, 1998. Smads: Transcriptional activators of TGF-beta responses. Cell 95(6). Dec. 11: 737-740.

Heldin, C.H., K. Miyazono, T. D. Peter, 1997. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. Nature, 390 : 465-471.

Labbe, E., C. Silvestri, P. A. Hoodless, J. L. Wrana, L. Attisano, 1998. SMAD2 and SMAD3 positively and negatively regulate TGFbeta-dependent transcription through the forkhead DNA-binding protein FAST2. Molecular Cell, 2: 109-112.

Lagna, G., A. Hata, B. A. Hemmati, J. Massague, 1996. Partnership between DPC4 and SMAD proteins in TGF-beta signalling pathways. Nature, 383 : 832-836.

Liu, F., C. Pouponnot, J. Massague, 1997. Dual role of the SMAD4/DPC4 tumor suppressor in TGFbeta-inducible transcriptional complexes. Genes & Development, 11: 3157-3167.

Paterson J.W. ,1998. BRCA1: a review of structure and putative functions. Dis Markers., Feb., 13: 261-274.

Plath, A.,R., Elinspanier, F.Peters, F. Sinowatz and D. Schams, 1997. Expression of transforming growth factors alpha and beta-1 messenger RNA in the bovine mammary gland during different stages of development and lactation. J. Endocrinol., 155:501.

Plaut, K., 1993 Role of epidermal growth factor and transforming growrth factors in mammary development and lactation. J. Dairy Sci., 76:1526

Robinson, S.D.,G.B. Silberstein, A.B. Roberts, K.C. Flanders, and C.W. Daniels, 1991. Regulated expression of and growth inhibitory effects of transforming growth factor-β isoforms in mouse mammary gland development (CAMB) 113:867.

Silberstein, G. B. and C.W. Daniel, 1987. Reversible inhibition of mammary gland growth by transforming growth factor-β. Science, 237:291.

Woodward, T.E., W.E. Beal and R.M. Akers, 1993. Cell interactions in initiation of mammary epithelial proliferation by estradiol and progesterone in prepubertal heifers. J. Endocrinol., 136:149.

Zawel-Leigh, Le-Dai-Jia., P. Buckhaults, Zhou-Shibin, W. Kenneth Kinzler, Bert Vogelstein and. Scott E. Kern, 1998. Human Smad3 and Smad4 are sequence specific transcription activators. Molecular Cell, 1: 611-617.

Zhang, Y., X. H. Feng, R. Y. Wu and R. Derynck, 1996, Receptor-associated Mad homologues synergize as effectors of the TGF-beta response. Nature, 383, 168-172.

Zhang-Ying. Feng-Xin-Hua and Derynck-Rik, 1998. SMAD3 and SMAD4 cooperate with c-jun/c-fos to mediate TGF-beta- induced transcription. Nature, 394: 909-913.

Abreviation key: TGF = transforming growth factor beta, BME = bovine mammary epithelial cells, BRCA1 = breast ovarian cancer susceptibility protein 1, SMADS = signaling effectors.