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Effects of BST and high energy diet on gene expression in mammary parenchyma of dairy heifers

Betina Joyce Lew[1], Mauro Dal Secco de Oliveira[2], José Esler de Freitas Júnior[3], Marina Vieira de Carvalho[3], Aníbal Coutinho do Rêgo[2], Francisco Palma Rennó[3]

[1] Department of Animal Science, Michigan State University, East Lansing, MI, USA.
[2] Departamento de Zootecnia, Universidade Estadual Paulista "Júlio de Mesquita Filho" - UNESP, Via de Acesso Prof. Paulo Donato Castellane s/n, CEP 14884-900, Jaboticabal, SP, Brazil.
[3] Departamento de Produção e Nutrição Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo (USP), Avenida Duque de Caxias Norte, 225 - Campus da USP, CEP: 13635-900, Pirassununga, SP, Brazil.

ABSTRACT - The objective of this study was to determine the effects of dietary energy and recombinant bovine somatotropin (bST) injection to identify genes that might control mammogenesis. Total RNA was extracted from the parenchymal tissue of 32 heifers randomly assigned to one of four treatments: two diets (a standard diet and a high energy, high protein diet), each with or without bST. To perform microarray experiments, RNA samples were pooled (2 animals/pool) before reverse transcription and labeling with Cy3 or Cy5. A 4-node loop design was used to examine the differential gene expression among treatments using a bovine-specific cDNA microarray (National Bovine Functional Genomics Consortium Library, NBFGC) containing 18,263 unique expressed sequence tags (EST). Significance levels of differential gene expression among treatments were assessed using a mixed model approach. Injection of bST altered the expression of 12 % of the genes on NBFGC slide related to tissue development, whereas 6% were altered by diet. Administration of bST increases the expression of genes positively related to cell proliferation and mammary parenchyma to a greater extent than a high energy diet.

Key Words: gene regulation, mammary gland development, microarray, nutrition, somatotropin

Introduction

The establishment of milk yield potential is critically determined during the prepubertal phase of mammary development (Sejrsen & Purup, 1997). A complex process involving environmental factors such as photoperiod and diet, the endocrine system, and stimulatory and inhibitory autocrine/paracrine factors regulates mammary development (Forsyth, 1989). It is well accepted that nutrition plays an important role in mammary development and subsequent milk production. Several reports have shown that feeding heifers with high-energy diets during the prepubertal period is detrimental to mammary development, with subsequent permanent reduction in milk production (Sejrsen & Purup, 1997). However, Radcliff et al. (1997) did not find a decrease in mammary parenchymal tissue mass or DNA when prepubertal heifers were fed high energy diets combined with high protein levels. In a subsequent experiment, animals fed the same prepubertal diet produced 14% less milk during first lactation when compared with animals receiving a standard diet (Radcliff et al., 2000).

Administration of bST to prepubertal dairy heifers increases growth rates and decreases carcass fat (Bauman et al., 1991; Vestergaard et al., 1993). In mammary tissue, bST treatment increases the total mass of parenchyma, and the amount of parenchymal DNA and RNA decreases the mass of adipose tissue (Sejrsen et al., 1986; Radcliff et al., 1997) Despite the many studies that have examined effects of diet or bST on mammary development, very little is understood about the possible mechanisms that mediate effects of heifer management on mammary cell proliferation and subsequent milk production. The development of microarray technology (Suchyta et al., 2003) has made it possible to examine a large number of genes that are being differentially expressed in different situations. Identification of new genes related to cell proliferation can lead to a better characterization of autocrine and paracrine factors involved in the process of mammary gland development.

The objective of the present study was to determine the effects of feeding a diet with increased energy and protein with or without bST injection on the gene expression profile of mammary parenchymal tissue of prepubertal heifers and to identify genes that control mammogenesis, and which can be altered by these managements.

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Corresponding author: joseeslerfjr@usp.br

Material and Methods

The tissue used in the present study was collected in a previous experiment conducted in 1994 at Michigan State University. Animal management, diet and tissue collection are described in detail in Radcliff et al. (1997). Briefly, thirty-two Holstein heifers, average body weight (BW) of 126 kg, were randomly assigned to one of four treatments from 4 months of age until slaughter at the luteal phase of the fifth estrous cycle, which is on average 71 days after puberty onset. Low-control (LC) animals were fed a total mixed diet formulated to produce an average daily gain of 0.8 kg BW/d. High-control (HC) animals were fed a total mixed diet with elevated protein and energy formulated to produce an average daily gain of 1.2 kg BW/d. Low-bST (LB) animals were fed the low-control diet and received a daily injection of 25 µg of bST/kg of BW (Pfizer Animal Health, Pharmacia and Upjohn Inc., Kalamazoo, MI), and high-bST (HB) animals were fed the high energy diet and daily injected 25 µg of bST/kg of BW. All injections were intramuscular. At slaughter, samples of mammary parenchyma and mammary adipose tissue were collected and stored at -80 ºC.

Total RNA was isolated from 100 mg parenchymal tissue of 32 animals (8/treatment) using Ribopure kit (Ambion). After isolation, the RNA concentration was determined by measuring absorbance at 260 nm; RNA solution was precipitated for at least one hour at -20 ºC and washed with 75% ethanol. Dry pellet of RNA was then suspended in a previously calculated volume of RNAse-free MiliQ water to reach a minimum concentration of 1.25 µg/µl, based on first spectrophotometer measurements. A new spectrophotometer evaluation was performed in order to check final concentration of RNA. To check quality, 1.0 µl of RNA sample containing 100 to 500 ng RNA was analyzed with Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA), and RNA quality was considered acceptable if the ratio between peaks for the 28 S and 18 S rRNA was ≥ 0.9. Samples were then stored at -80 °C until microarray analysis was performed.

The experiment was conducted in a 2 × 2 factorial arrangement, with two different diets (low or high energy and protein levels, denoted by L and H respectively), with or without bST (represented by B and C, respectively). Total RNA samples were pooled (2 samples/pool) to generate a total of 16 pooled samples, four per treatment. The microarray experiment consisted of four independent loops, each with different ordering and labeling of treatments so that all six possible different combinations (LC × HC; LC × LB; HC × HB; LB × HB; LC × HB, HC × LB) were represented at least once in both directions.

The National Bovine Functional Genomic Consortium (NBFGC) library containing 18,263 unique expressed sequence tags (EST) was used for microarray experiments. Library description and microarray protocol were previously detailed by Suchyta et al. (2003). Total RNA (10 µg) was used as template in reverse transcription reaction with Atlas PowerScript Fluorescence Labeling system (BD Biosciences, Alameda, CA) with oligo $(dT)_{18}$ as primer. Following first-strand synthesis and prior to hybridization, cDNA were labeled using n-hydroxisuccinate (NHS)-derivatized Cy3 and Cy5 dyes (Amersham Bioscencies). The hybridization process was performed at GeneTAC Hybridization Station (Genomic Solutions, Ann Arbor, MI), with temperatures varying from 42 to 65 ºC, for 18 hours in vacuo. After hybridization and washing with medium and high stringency buffer (Genomic Solutions), slides were rinsed briefly at room temperature in 2 X SSC and 1 X in double-distilled H_2O and dried by centrifugation at 300 × *g* at room temperature for 2 min inside 50 mL conical tubes. Dried slides were scanned immediately in a GeneTAC LS IV microarray scanner (Genomic Solutions).

GeneTAC integrator 4.0 software was used to process array images, align spots, and integrate robot-spotting files with the microarray images and export intensity data. The final report was retrieved as raw spot intensities in comma-separated value files, compatible with Microsoft Excel and SAS (Statistical Analysis System, version 8).

Array-specific data normalization was performed using a robust local regression technique (Cleveland & Grosse, 1991) with the LOESS procedure of SAS (Statistical Analysis System, version 8). The efficiency of LOESS normalization was assessed by monitoring *Mus-A* plots (Yang et al., 2002) and log *Cy*3 versus log *Cy*5 scatter plots for data from each array before and after normalization. The normalized data were then back transformed prior to further statistical analyses using the formula: log $Cy3^* = A + M^* / 2$ and log $Cy5^* = A - M^* / 2$, where log *Cy*3* and log *Cy*5* are the normalized log intensities. Here, $M^* = M - \hat{M}$ represents each of the normalized *M* values, with $\hat{M}$ = LOESS predicted value for each spot. Log intensities adjusted via LOESS were then analyzed statistically using a mixed model approach consisting of two steps (Wolfinger et al., 2001). The first step involved array-specific spatial variability normalization and the second step, gene-specific analyses to test the effect of diet and bST on expression profiles for individual genes. The normalization model in the first step included the overall effects of treatments, arrays, pools, dye, and patch within array. The second step of the statistical analysis consisted of gene-specific models for the estimated residuals obtained from the normalization

approach described above. For each gene, a linear mixed model was considered, including the fixed effects of diet, bST, the interaction between them and the effect of dye, as well as the random effects of replication (pools), array and residual effects. The P-values from these tests were converted to q-values to establish statistical significance based on a false discovery rate (FDR) of 5% for multiple testing (Storey, 2002). The analyses were computed by using the MIXED procedure of SAS (Statistical Analysis System, version 8).

Next, the spotted cDNA sequences representing genes whose expression profiles varied significantly across time were subjected to BLASTN analysis to reveal identities, and the functions of these genes were determined through an extensive PubMed literature search. This information was used to form preliminary clustering of affected genes into broad functional categories for presentation in the results.

Results and Discussion

Heifers in groups LC and LB were slaughtered approximately 276 d after the beginning of the experiment, while animals in groups HC and HB were slaughtered after around 218 d. Treatments resulted in gains of 0.77 kg BW/d (group LC); 0.85 kg BW/d (group LB); 1.19 kg BW/d (group HC) and 1.27 kg BW/d (group HB).

Within main contrasts (B × C, H × L), 1083 genes were differentially expressed (P<0.05). The bST injection altered the expression of 620 genes, while high energy and-protein diet altered the expression of 463 genes (P<0.05). There are multiple ways to explore biological significance of results obtained in microarray experiments. One option is to organize the genes into functional groups of signaling pathways, to examine the regulation of clusters of genes. The authors of the present study aimed at identifying bST and/or high-energy diet-regulated genes, involved in the induction or inhibition of parenchymal tissue development.

Four hundred and forty-eight genes in NBFGC library were putatively identified as related to inhibition of tissue development, according to their biological or molecular functions in gene ontology (GO) data base. Genes with function related to cell cycle, cell adhesion, cell proliferation and cell growth were considered positively related to tissue development (proliferative genes), while genes related to cell death, apoptosis, cell cycle arrest and negative regulation of cell proliferation were considered inhibitors of tissue development (anti-proliferative genes).

Regardless of diet, bST injection altered the expression of 53 (11.8%) genes related to tissue development, up-regulating the expression of 34 proliferative genes (Tables 1 and 2) and only two (DEAD-box protein abstrakt (ABS) and Growth-arrest specific 8) anti-proliferative genes (P<0.05) (Table 3). Seventeen genes were down-regulated by the bST treatment (P<0.05; Tables 1, 2 and 3); six of them were classified as anti-proliferative genes (insulin-like growth factor binding protein 3 - IGFBP3; caspase recruitment domain family, member 11; inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma; interleukin 2 receptor; and interleukin 18BRCA1-associated protein-1 (ubiquitin carboxy-terminal hydrolase)) (Table 3).

On the other hand, only 26 (5.7%) genes related to tissue development were altered by diet, stimulating the expression of 14 proliferative genes (Table 4) and two anti-proliferative genes (Table 5); and inhibiting the expression of 10 proliferative genes (Table 4; Figure 1).

Radcliff et al. (1997) reported that administration of bST increased parenchymal weight and DNA content of the mammary gland by 47%, when compared with non-injected control animals, suggesting that bST stimulates proliferation of mammary parenchymal cells. In the present work, several novel bST-regulated proliferative genes were identified (Tables 2 and 3).

In the present study, bST increased mRNA for insulin-like growth factor 1 (IGF-I) in liver and serum concentration of IGF-1, as previously reported by Radcliff et al. (2004), but did not alter IGF-1 mRNA in the parenchymal tissue. Treatment with bST reduced the abundance of mRNA for IGFBP3 in mammary tissue (P<0.01), which is consistent with previous findings (Berry et al., 2001).

Since IGFBP3 is negatively associated with cell proliferation (Huynh et al., 1996) down-regulation of IGFBP3 mRNA could increase the number of epithelial cells, because it would increase the amount of IGF-I available to the epithelial cell receptors. Moreover, an IGF-1

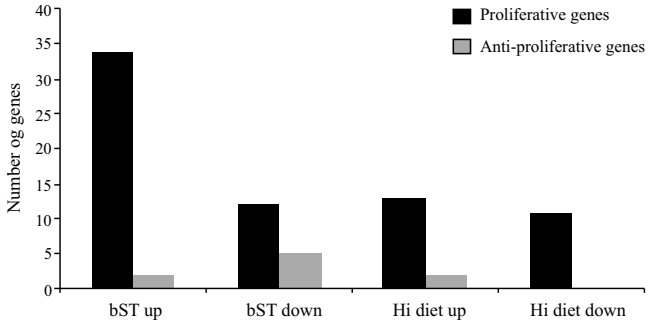

Figure 1 - Number of proliferative and anti-proliferative genes altered by diet and bST administration (P<0.05).

Table 1 - Gene ontology function and name of proliferative genes related to cell proliferation altered by bST administration

Sequence[1]	Gene[2]	B × C variation[3]	Mean				SEM	P-value[4]		
			LC	LB	HC	HB		bST	Diet	Inter
	Cell proliferation									
NBFGC_BG690462	Tumor protein D52-like 2	2.18	-0.206	0.581	-0.384	1.072	0.56	0.025	0.718	0.442
NBFGC_BF606672	Insulin-like growth factor binding protein, acid labile subunit	1.27	-1.916	-1.745	-1.984	-1.466	0.37	0.021	0.496	0.193
NBFGC_BG688237	Hepatoma-derived growth factor (high-mobility group protein 1-like)	1.20	2.263	2.348	2.294	2.747	0.22	0.049	0.122	0.154
NBFGC_AW660490	RAB26, member RAS oncogene family	1.18	-1.005	-0.756	-0.940	-0.719	0.16	0.043	0.676	0.893
NBFGC_BF073564	Glutamyl aminopeptidase (aminopeptidase A)	1.15	0.219	0.340	0.048	0.333	0.17	0.049	0.415	0.386
NBFGC_BE479946	Peroxiredoxin 1	1.11	1.532	1.579	1.393	1.658	0.23	0.025	0.638	0.093
NBFGC_BE755280	RAB37, member RAS oncogene family	1.10	1.273	1.371	1.382	1.519	0.22	0.030	0.048	0.977
NBFGC_BF601615	THO complex 2	-1.14	0.631	-0.962	-0.663	-0.705	0.41	0.011	0.117	0.037
NBFGC_BF230787	RAN, member RAS oncogene family	-1.17	0.030	-0.278	0.179	-0.032	0.33	0.028	0.062	0.835
NBFGC_BF652964	Tetraspan 2	-1.35	1.572	-2.134	-1.620	-1.921	0.21	0.014	0.629	0.384
	Cell growth									
NBFGC_BG688950	Ubiquitin specific protease 4 (proto-oncogene)	2.31	0.680	1.571	1.194	2.716	0.54	0.031	0.673	0.757
NBFGC_BE684128	Fer (fps/fes related) tyrosine kinase (phosphoprotein NCP94)	1.18	0.332	0.551	0.393	0.655	0.44	0.007	0.349	0.760
NBFGC_BG691581	Vav 1 oncogene	-1.08	0.216	0.114	0.255	0.123	0.20	0.031	0.637	0.757
NBFGC_BE480828	Chromosome 10 open reading frame 6	1.88	1.065	1.967	1.532	2.446	0.36	0.021	0.204	0.987
NBFGC_BE485484	Hepsin (transmembrane protease, serine 1)	1.58	-0.064	0.244	-0.475	0.544	0.52	0.011	0.830	0.122
NBFGC_BE810065	Collagen, type XI, alpha 2	1.37	-2.425	-2.011	-2.523	-2.020	0.29	0.005	0.689	0.727
NBFGC_BE589677	Transforming growth factor, beta receptor III (betaglycan, 300kda)	1.33	-1.456	-0.988	-1.209	-0.858	0.30	0.020	0.302	0.695
NBFGC_AW336151	Novel protein similar to SEL1L (sel-1 (suppressor of lin-12, C.elegans)-like)	1.21	-0.944	-0.863	-1.091	-0.612	0.31	0.028	0.699	0.096
NBFGC_BE846147	Annexin A2	1.20	2.274	2.550	2.428	2.681	0.29	0.035	0.253	0.915
NBFGC_BG690462	Putative translation initiation factor	1.15	-0.747	-0.534	-0.955	-0.767	0.29	0.050	0.067	0.894
NBFGC_AW426175	Annexin VIII	1.13	-0.377	-0.362	-0.369	-0.030	0.12	0.046	0.072	0.063
NBFGC_AW429437	Heparanase	-1.14	1.805	-2.150	-1.735	-1.761	0.24	0.041	0.028	0.071

[1] Gene sequence in the NBFGC library (http://gowhite.ans.msu.edu/public_php/NBFGC_GeneLink.html).
[2] Genes according to their biological classification in Gene Ontology (http://www.godatabase.org/cgi-bin/amigo/go.cgi).
[3] Variation between the groups receiving (HB + LB) and not receiving bST (HC + LC), calculated based on the average of 2 treatments raised to the exponential.
[4] Level of significance for effects of bST, diet, and interaction (Inter) between diet and bST with significant effect at $P<0.05$.
LC - control diet, no bST; LB - control diet with bST administration; HC - high dietary energy and protein without bST; HB - high dietary energy and protein, with administration of bST; SEM - standard error of the mean.

Table 2 - Gene ontology function and name of proliferative altered by bST administration

Sequence[1]	Gene[2]	B × C variation[3]	Mean				SEM	P-value[4]		
			LC	LB	HC	HB		bST	Diet	Inter
	Cell cycle									
NBFGC_BE663477	Sirtuin (silent mating type information regulation 2 homolog) 2 (S. Cerevisiae)	1.29	-2.540	-2.367	-2.740	-2.175	0.19	0.001	0.953	0.011
NBFGC_AW481824	Cyclin D1 (PRAD1: parathyroid adenomatosis 1)	1.27	-0.859	-0.607	-1.141	-0.695	0.31	0.006	0.143	0.342
NBFGC_BF230594	TNF receptor-associated factor 6	1.27	-2.672	-2.316	-2.572	-2.231	0.33	0.018	0.543	0.952
NBFGC_BE809775	Homo sapiens pleckstrin homology domain containing, family B (evectins) member 1 (PLEKHB1), mRNA	1.23	-0.708	-0.350	-0.857	-0.621	0.25	0.043	0.169	0.641
NBFGC_BE754205	PECAM 1	1.22	-0.893	-0.741	-1.126	-0.709	0.19	0.043	0.441	0.303
NBFGC_BG689207	Spectrin, alpha, non-erythrocytic 1 (alpha-fodrin)	1.20	2.701	2.856	2.760	3.123	0.35	0.032	0.163	0.335
NBFGC_BF605019	Microtubule-associated protein 1B	1.17	-0.310	-0.214	-0.296	0.061	0.18	0.023	0.149	0.149
NBFGC_BF654231	Histidine triad nucleotide binding protein 1	1.15	-0.176	-0.159	-0.278	0.121	0.13	0.021	0.287	0.031
NBFGC_BE722663	Homo sapiens PAC clone RP5-886O8 from 7, complete sequence	1.15	0.033	0.291	0.061	0.207	0.34	0.028	0.773	0.487
NBFGC_BG691656	Cyclin G1	1.15	1.329	1.433	1.211	1.499	0.13	0.009	0.703	0.151
NBFGC_AW656282	Transmembrane, prostate androgen induced RNA	1.14	0.291	0.428	0.382	0.621	0.12	0.013	0.064	0.426
NBFGC_BE808490	CHK2 checkpoint homolog (S. Pombe)	1.13	0.833	0.961	0.834	1.053	0.17	0.047	0.619	0.559
NBFGC_BE476311	Baculoviral IAP repeat-containing 6 (apollon)	-1.17	0.416	0.258	0.442	0.151	0.31	0.039	0.724	0.493
NBFGC_845840	Platelet-activating factor acetylhydrolase, isoform Ib, alpha subunit 45kda	-1.18	1.323	-1.395	-1.153	-1.548	0.10	0.017	0.929	0.076
	Cell adhesion									
NBFGC_BG691961	Catenin (cadherin-associated protein), alpha 1, 102kda	2.07	0.402	1.163	0.432	1.776	0.375	0.012	0.373	0.405
NBFGC_BE588613	Collagen, type XIV, alpha 1 (undulin)	1.92	-0.248	0.447	-0.085	1.103	0.567	0.027	0.291	0.507
NBFGC_BE589641	Chemokine (C-X-C motif) receptor 3	1.73	-1.014	-0.431	-0.854	0.139	0.413	0.043	0.316	0.556
NBFGC_BE480007	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	1.13	-1.482	-1.365	-1.532	-1.308	0.298	0.023	0.964	0.415
NBFGC_BE230242	Vascular cell adhesion molecule 1	1.09	0.443	0.458	0.383	0.620	0.134	0.042	0.446	0.066
NBFGC_BE683087	RIKEN	-1.10	0.937	0.815	0.933	0.771	0.179	0.030	0.729	0.723
NBFGC_BE808135	Integrin, alpha 7	-1.14	0.909	0.785	0.925	0.661	0.173	0.039	0.545	0.408
NBFGC_AW660248	Immunoglobulin superfamily containing leucine-rich repeat	-1.21	0.549	-0.919	-0.901	-1.078	0.372	0.046	0.099	0.436
NBFGC_AW307628	Fibulin 5	-1.27	0.411	-0.536	-0.467	-1.038	0.184	0.050	0.118	0.180

[1] Gene sequence in the NBFGC library (http://gowhite.ans.msu.edu/public_php/NBFGC_GeneLink.html).
[2] Genes according to their biological classification in Gene Ontology (http://www.godatabase.org/cgi-bin/amigo/go.cgi).
[3] Variation between the groups receiving (HB + LB) and not receiving bST (HC + LC), calculated based on the average of 2 treatments raised to the exponential.
[4] Level of significance for effects of bST, diet, and interaction (Inter) between diet and bST with significant effect at P<0.05.
LC - control diet, no bST; LB - control diet with bST administration; HC - high dietary energy and protein without bST; HB - high dietary energy and protein, with administration of bST; SEM - standard error of the mean.

Table 3 - Anti-proliferative genes altered by bST administration

Sequence[1]	Gene[2]	B × C variation[3]	Mean				SEM	P-value[4]		
			LC	LB	HC	HB		bST	Diet	Inter
	Celular apoptose									
NBFGC_AW654871	BRCA1 associated protein-1 (ubiquitin carboxy-terminal hydrolase)	-1.19	0.466	0.279	0.518	0.197	0.25	0.010	0.864	0.415
NBFGC_BE482798	growth arrest-specific 8	2.02	0.955	1.665	1.068	2.381	0.51	0.047	0.380	0.512
NBFGC_BE752216	Caspase recruitment domain family, member 11	-1.28	0.659	-1.225	-0.758	-0.899	0.30	0.033	0.508	0.165
NBFGC_BF652935	Interleukin 18 (interferon-gamma-inducing factor)	-1.31	2.074	-2.535	-1.904	-2.219	0.38	0.034	0.224	0.648
NBFGC_BE756772	Interleukin 2 receptor	-1.36	1.200	-1.442	-0.815	-1.463	0.41	0.022	0.321	0.239
NBFGC_BE752722	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma	-1.30	1.645	-2.253	-1.831	-1.973	0.38	0.046	0.813	0.184
NBFGC_BG693382	DEAD (Asp-Glu-Ala-Asp) box polypeptide 41	1.80	1.840	2.459	1.714	2.789	0.35	0.020	0.753	0.469
NBFGC_BF654852	*Bos taurus* insulin-like growth factor binding protein-3 (IGFBP-3) gene, complete cds	-1.31	1.674	-2.113	-1.507	-1.850	0.21	0.005	0.118	0.670

[1] Gene sequence in the NBFGC library (http://gowhite.ans.msu.edu/public_php/NBFGC_GeneLink.html).
[2] Genes according to their biological classification in Gene Ontology (http://www.godatabase.org/cgi-bin/amigo/go.cgi).
[3] Variation between the groups receiving (HB + LB) and not receiving bST (HC + LC), calculated based on the average of 2 treatments raised to the exponential.
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independent effect was also postulated for IGFBP3 (Akers et al., 2000), since the addition of this protein blocked the mitogenic effects of mammary extracts, thus a direct effect of the decrease in IGFB3 should be considered.

Somatotropin altered the expression of 9 genes related to cell adhesion. Cell adhesion (Table 2) primarily depends on the cadherin and integrin families of molecules, acting on segregation, sorting, rearrangements and migration of cell populations, and providing the basis for the dynamic morphogenetic processes of tissue formation (Steinberg, 1996). In the mammary gland, the epithelial type of adherent junction is composed of the E-cadherin/catenin complex. This complex consists of transmembrane E-cadherin and its associated intracellular catenins (α, β, γ and p120). The extracellular region of E-cadherin is responsible for homotypic interactions facilitating cell-cell connections, while the cytoplasmic domain of E-cadherin binds to β-catenin. Beta-catenin binds to α-catenin, which is required to anchor the cadherin complex to the actin cytoskeleton (Beavon, 2000).

The mammary gland expresses α-catenin during all stages of development, and the biogenesis of a functional mammary gland depends on the presence of this molecule. Alpha-Catenin is needed for normal mammary cell adhesion, polarization, stabilization and functioning (Nemade et al., 2004). In the present study, bST increased the expression of α-catenin mRNA by 100%, compared to bST control animals (P<0.02; Table 2). One reason for the increased α-catenin might be the involvement of this molecule in IGF-1-induced cell-migration (André et al., 2004); conversely, α-catenin might be directly involved in the IGF-1 signaling pathway, since the loss of α-catenin alters the response of keratinocytes to several growth factors (Vasioukhin et al., 2001).

Interestingly, the increase in α-catenin mRNA was coincident with an increase in the platelet-endothelial-cell adhesion molecule (PECAM-1) (P<0.05; Table 2). PECAM-1 is a glycoprotein belonging to the immunoglobulin superfamily of cell-adhesion molecules expressed in endothelial cells, platelets and specific cells of the immune system (Ilan & Madri, 2003). Lack of PECAM-1 expression in transgenic virgin mice led to impairment in mammary ductal branching morphogenesis and decrease in ductal epithelial cell proliferation (Ilan et al., 2001). Evidence suggests that this effect occurs through interactions with molecules of the STAT system and catenin complex (Ilan et al., 2001). Moreover, PECAM-1 promoted β-catenin accumulation and stimulated cell proliferation (Biswas et al., 2003). The findings of our work, demonstrating that bST-treated animals had increased expression of both PECAM-1 and α-catenin

Table 4 - Proliferative genes altered by diet

Sequence[1]	Gene[2]	B × C variation[3]	Mean				SEM	P-value[4]		
			LC	LB	HC	HB		bST	Diet	Inter
	Cell proliferation									
NBFGC_AW645771	Heparin binding protein-44 [mice, mrna, 1478 nt]	-1.09	0.035	0.026	-0.074	-0.105	0.18	0.596	0.024	0.767
NBFGC-AW654906	Integrin, beta 2 (antigen CD18 (p95)	1.19	-1.162	-1.152	-0.880	-.0921	0.19	0.813	0.008	0.693
NBFGC_BE484656	Tumor protein D52-like 2	1.20	1.595	1.758	2.002	1.881	0.28	0.839	0.038	0.189
NBFGC_BE751204	Misshapen/NIK-related kinkinase (MINK)	1.31	-1.883	-2.087	-1.660	-1.520	0.25	0.781	0.017	0.162
NBFGC_BF074683	Acetylcholinesterase	1.13	0.096	0.002	0.265	0.182	0.24	0.245	0.043	0.944
NBFGC_BG690060	Ferritin, heavy polypeptide 1	-1.26	-0.534	-0.285	-0.803	-0.678	0.22	0.162	0.045	0.627
	Cell growth									
NBFGC_AW429437	Heparanase	1.17	-1.805	-2.150	-1.735	-1.761	0.24	0.041	0.028	0.071
NBFGC_BE590166	5'-3' exoribonuclease 2	1.31	-2.128	-2.026	-1.529	-1.836	0.35	0.476	0.023	0.170
NBFGC_BF193944	Sushi-repeat protein (SRPUL)	-1.34	-1.191	-1.250	-1.612	-1.672	0.13	0.394	0.001	0.996
NBFGC_BF602183	Platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog) (PDGFB)	-1.10	3.200	3.179	3.113	2.996	0.21	0.245	0.042	0.408
NBFGC_BF776829	Insulin-like growth factor 2 receptor	1.17	-0.056	0.008	0.179	0.224	0.14	0.372	0.011	0.872
NBFGC_BF889524	Interferon regulatory factor 1	1.36	-1.451	-1.515	-0.966	-1.107	0.22	0.532	0.036	0.812
NBFGC_BG692794	Colon cancer, nonpolyposis type 2 (MLH1)	1.22	-0.260	-0.166	-0.026	0.169	0.18	0.082	0.010	0.510
NBFGC_AW660002	Tropomyosin 3	-1.11	-0.053	-0.148	-0.198	-0.299	0.19	0.088	0.042	0.948
	Cell cycle									
NBFGC_AW345640	Deoxythymidylate kinase	1.18	-1.038	-0.998	-0.895	-0.653	0.29	0.132	0.019	0.265
NBFGC_AW658462	Regulator of G-protein signaling 2	-1.29	0.058	0.082	-0.244	-0.353	0.26	0.710	0.022	0.566
NBFGC_BE683116	Fibroblast growth factor receptor 2	1.11	0.771	0.931	1.079	0.909	0.28	0.781	0.020	0.010
NBFGC_BE753819	Latent transforming growth factor beta binding protein 2 (LTBP2)	1.12	-0.233	-0.078	-0.023	0.036	0.12	0.096	0.044	0.425
	Cell adhesion									
NBFGC_AW357603	Collagen, type IV, alpha	-1.24	-1.755	-1.588	-1.953	-2.017	0.29	0.634	0.036	0.299
NBFGC_AW484319	Myeloid/lymphoid	1.23	-2.178	-2.205	-1.978	-1.807	0.21	0.436	0.020	0.292
NBFGC_AW659967	Discoidin domain receptor	-1.21	-0.361	-0.457	-0.562	-0.806	0.21	0.120	0.033	0.471
NBFGC_AW669921	Carbohydrate sulfotransferase	1.41	-2.504	-2.672	-1.871	-2.315	0.19	0.069	0.018	0.376
NBFGC_BF076192	Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein) (CEACAM1)	-1.26	-0.421	-0.550	-0.708	-0.931	0.19	0.215	0.041	0.728
	Cell motility									
NBFGC_BE757172	Autocrine motility factor	-1.22	-1.489	-1.502	-1.713	0.348	0.49	0.026	0.422	0.422

[1] Gene sequence in the NBFGC library (http://gowhite.ans.msu.edu/public_php/NBFGC_GeneLink.html).
[2] Genes according to their biological classification in Gene Ontology (http://www.godatabase.org/cgi-bin/amigo/go.cgi).
[3] Variation between the groups receiving (HB + LB) and not receiving bST (HC + LC), calculated based on the average of 2 treatments raised to the exponential.
[4] Level of significance for effects of bST, diet, and interaction (Inter) between diet and bST with significant effect at P<0.05.
LC - control diet, no bST; LB - control diet with bST administration; HC - high dietary energy and protein without bST; HB - high dietary energy and protein, with administration of bST; SEM - standard error of the mean.

Table 5 - Anti-proliferative genes altered by diet

Sequence[1]	Gene[2]	B × C variation[3]	Mean				SEM	P-value[4]		
			LC	LB	HC	HB		bST	Diet	Inter
Celular apoptosis										
NBFGC_AW484298	Leucine-rich repeat LGI3	1.13	-0.039	-0.054	0.137	0.155	0.320	0.012	0.937	0.937
NBFGC_AW654346	Myeloid membrane glycoprotein precursor (CD14)	1.22	-0.424	-0.306	0.004	-0.152	0.133	0.850	0.028	0.187

[1] Gene sequence in the NBFGC library (http://gowhite.ans.msu.edu/public_php/NBFGC_GeneLink.html).
[2] Genes according to their biological classification in Gene Ontology (http://www.godatabase.org/cgi-bin/amigo/go.cgi).
[3] Variation between the groups receiving (HB + LB) and not receiving bST (HC + LC), calculated based on the average of 2 treatments raised to the exponential.
[4] Level of significance for effects of bST, diet, and interaction (Inter) between diet and bST with significant effect at P<0.05.
LC - control diet, no bST; LB - control diet with bST administration; HC - high dietary energy and protein without bST; HB - high dietary energy and protein, with administration of bST; SEM - standard error of the mean.

mRNA, suggest a novel mechanism for somatotropin actions in mammary development involving those molecules.

Another interesting molecule, stimulated by bST administration, was serine protease hepsin (Table 1) (P<0.02). Hepsin is a type II transmembrane serine protease found in significant levels in many different types of mammalian cells (Tsuji et al., 1991; Torres-Rosado et al., 1993). Proteases are present during cell migration and tissue rearrangement during morphogenesis, creating space for cell migration and promoting extension of epithelial cells through the extracellular matrix (Saksela & Rifkin, 1988).

Serine proteases are known to have growth factor-like activity (Fenton, 1986) and hepsin seems to be indispensable for cell growth and for maintenance of normal cell morphology (Torres-Rosado et al., 1993).

The increase in hepsin mRNA, observed in this study, suggests that this serine protease is somehow involved in bST-stimulation of mammary parenchyma development. The mechanisms by which proteases stimulate cell growth and proliferation are not totally clear. Other proteases, such as the prostate specific-antigen (PSA), were shown to be involved in IGF-I pathway by cleaving the IGFBP3 molecule and lowering its affinity for IGF-1, allowing IGF-1 to bind to its membrane receptor on benign prostate hyperplasia (BPH) epithelial cells (Cohen et al., 1992). Moreover, Sutkowski et al. (1999) found that proteases attenuated the inhibitory effects of IGFBP-3 in BPH-derived stromal cells *in vitro*, and PSA stimulated the growth of those cells by 17%.

An alternative proliferative response might result from protease binding to specific cell-surface receptors (Vu et al., 1991). Proteases may also work as autocrine growth factors on the surrounding responsive epithelial cells, as *in vitro* experiments have demonstrated both direct and indirect growth responses in normal and neoplasic prostate epithelial cells to proteases such as PSA (Cohen et al., 1992). Overexpression of hepsin by bST injection suggests that this protease might participate in stimulation of mammary parenchymal growth.

In addition, somatotropin injection up-regulated the expression of several genes involved in cell proliferation (such as the tumor protein D52 and the hepatoma-derived growth factor), two different types of collagen, genes involved in cell adhesion (Table 1), genes involved in cell cycle progression, such as cycle D1 and cycle G1 (Table 2), proteases involved in the acceleration of the G1 phase of the cell cycle, and in the transition grom the G1 to the S phase of the cell cycle (Motokura et al., 1991). Somatotropin also down-regulated the expression of some genes related to

apoptosis such as interleukin 18 and interleukin 2 receptor (Table 3).

High-energy diet altered the expression of only 5.7% of the genes in NBFGC library, related to tissue development ($P<0.05$) (Tables 4 and 5). However, the NBFGC library evaluates more than 18,000 genes at a time, and may not be so efficient in the detection of specific genes alterations. Therefore, a possibly higher effect of diet should not be totally discarded, and it is important that future studies apply more precise techniques to evaluate more subtle variations that may have not been identified.

Results of this study suggest that there was no detrimental effect of high-energy high-protein diet on mammary parenchyma development, and Radcliff et al. (1997), analyzing the same samples, reported that the high-energy diet did not reduce cell number or metabolic activity of the mammary gland.

However, Davis Rinker et al. (2008) reported that increasing dietary energy intake of weaned prepubertal heifers inhibits mammary growth, relative to body growth, in a time-dependent manner, and when used for a longer duration, it linearly decreased the mass of fat-free mammary parenchymal tissue. The authors also observed a linear reduction in the percentage of proliferating epithelial cells, which is consistent with the idea that high energy diets reduce the mammary parenchymal mass at puberty. In this regard, Radcliff et al. (2000) observed a 14% reduction in milk production in the first lactation of heifers fed diets with high energy and high protein levels.

On the other hand, Brown et al. (2005) reported that increasing energy and protein intake in Holstein heifer calves from 2 to 8 weeks of age can increase the rate of mammary parenchyma development, but it is not clear if this would increase milk production later in life.

Conclusions

Treatment with bST alters the expression of several genes related to parenchymal development, including growth factors and molecules involved in the cell cycle, cell adhesion, cell-to-cell communication, components of extra cellular matrix, cell growth and cell proliferation. Future studies should explore the involvement of these genes in mediating the mammogenic response of the recombinant bovine somatotropin. In contrast, feeding a high energy and protein diet altered few genes in mammary parenchyma, consistent with the lack of a direct negative effect of high-energy treatment on mammary parenchyma development in these heifers.

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