Aromatase expression in the human fetal osteoblastic cell line SV-HFO

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Abstract

A number of clinical studies have highlighted the importance of estrogen in bone growth and maintenance in men and postmenopausal women. In these instances, estrogen is synthesized locally within bone tissue by aromatase, encoded by the CYP19 gene. The mechanisms regulating aromatase expression in bone, however, are unclear. In this work we characterized the expression of aromatase activity and gene transcripts in the human fetal osteoblastic cell line, SV-HFO. Aromatase activity and gene transcript expression were stimulated by dexamethasone. Oncostatin M strongly stimulated aromatase expression in synergy with dexamethasone. These factors induced CYP19 transcripts that included the sequence of exon I.4 in their 5′UTR. Consistent with this, a reporter construct harboring the genomic sequence of the promoter region of exon I.4 (promoter I.4) was also activated by dexamethasone and oncostatin M. 5′ deletion and mutation analysis revealed important roles for a glucocorticoid response element, an interferon γ activating sequence and a putative binding site for Sp1. Transfection of exogenous glucocorticoid receptor, STAT3 or Sp1 increased promoter activity, indicating a potential role for these transcription factors in regulating aromatase expression in SV-HFO cells. These data suggest that the SV-HFO cell line is a valuable model with which to elucidate the mechanisms regulating local estrogen synthesis in osteoblasts.

Journal of Molecular Endocrinology (2004) 32, 533–545

Introduction

Studies of human patients with estrogen deficiency have highlighted the importance of estrogen for pubertal bone growth and establishment of peak bone mineral density (BMD) (Grumbach 2000). Both male and female patients with defects in the estrogen receptor α (ERα) gene or the CYP19 gene that encodes aromatase show very low BMD, tall stature, delayed bone age, absence of epiphyseal closure and absence of the pubertal growth spurt (Smith et al. 1994, Morishima et al. 1995, Carani et al. 1997, Mullis et al. 1997, Bilezikian et al. 1998). Administration of estrogen to aromatase-deficient men resulted in increased BMD and epiphyseal closure (Carani et al. 1997, Mullis et al. 1997, Bilezikian et al. 1998). Knockout mouse models have supported the importance of estrogen in bone metabolism: male and female ERα (ERKO) and aromatase (ArKO) null mice exhibit low BMD (Oz et al. 2000, Vidal et al. 2000, Miyaura et al. 2001), and treatment of the ArKO mice with estrogen improved BMD (Miyaura et al. 2001). Recently, a significant relationship between a common CYP19 polymorphism (tetranucleotide tandem repeat in intron 4) and osteoporosis and fracture risk of postmenopausal women has been demonstrated (Masi et al. 2001).

immunoreactivity (Oz et al. 2001). The human CYP19 gene has multiple first exons, which are selected in a tissue-specific manner (Simpson et al. 1997, Sasano & Harada 1998, Sebastian & Bulun 2001) (Fig. 1). RT-PCR analysis indicates that the most dominant 5'UTR in aromatase gene transcript in human bone tissue is derived from upstream exon I.4 (Sasano et al. 1997, Shozu & Simpson 1998). This suggests that the 5' region of exon I.4 (promoter I.4) is predominantly used in bone tissue to drive aromatase expression.

The high levels of aromatase expression in these bone cells, together with the low circulating levels of estrogen in postmenopausal women and in men, lead to the conclusion that local aromatase expression in bone is the major source of estrogen acting on this tissue in these instances (Simpson & Davis 2001). The relationship between local aromatase expression and bone metabolism, however, is still not clear. An in vitro osteoblastic cell line would clearly be useful to define better the molecular mechanisms regulating aromatase expression and estrogen production in bone.

Recently, an SV40-immortalized human fetal osteoblastic cell line (SV-HFO) was established from human fetal calvaria (Chiba et al. 1993). SV-HFO cells exhibit alkaline phosphatase activity, osteocalcin gene expression and calcium deposition into extracellular matrix in response to dexamethasone (Dex) and β-glycerophosphate (β-GP) (Iba et al. 1995). Expression of aromatase, 17β-hydroxysteroid dehydrogenase and steroid sulfatase have also been reported in SV-HFO (Janssen et al. 1999). In addition, SV-HFO cells express both ERα and ERβ (Arts et al. 1997). These properties suggest that the SV-HFO cell line might be an ideal model with which to identify mechanisms of aromatase expression in osteoblasts. In the present study, we have defined the regulation of aromatase activity, expression of gene transcripts and promoter usage in SV-HFO cells by a variety of hormonal factors, including glucocorticoids and cytokines.

**Materials and methods**

**Materials**

Dexamethasone, forskolin, phorbol-12-myristate 13-acetate (PMA), cholecalciferol, recombinant human oncostatin M (OSM) and tumor necrosis factor α (TNFα) were from Sigma. Recombinant human bone morphogenetic protein-2, interleukin 1β, interleukin 6, interleukin 6 soluble receptor, and interleukin 11 were from R&D Systems (Minneapolis, MN, USA). [1β-3H] androstenedione was from Amersham Biosciences.

**Plasmids**

A luciferase reporter construct containing 1018 nucleotides of CYP19 promoter I.4 was prepared by PCR amplification of the appropriate region of genomic DNA and subcloning into the vector pGL3 (Promega). A series of 5'-deleted constructs were then prepared by PCR. Mutations of the GRE, GAS and Sp1 binding sites within the 1018 nt promoter were introduced using QuickChange (Stratagene) according to the manufacturer’s instructions. The specific mutations introduced were (mutated nucleotides in lower case) as follows: GRE, AGAAGATTCTGTTCT → ctgcagTTC ctgcag; GAS, TTCCTGTGAAA → TTCCTcTG cAg; Sp1, GGGCGGGGC → ctGCaGGGC.

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**Figure 1** Partial structure of CYP19 gene. Nomenclature of multiple exons I by Simpson and Harada (upper) (Sasano & Harada 1998, Simpson et al. 2002) and main sites of expression (lower) are indicated. Location of first exons is based on the analysis of human genome sequence by Sebastian and Bulun (2001).
Expression vectors encoding human STAT1, STAT3 and GRα were prepared by PCR amplification of the appropriate cDNA sequences from human adipose stromal cell cDNA and subcloning into the vector pCDNA3·1+ (Invitrogen). An expression plasmid encoding Sp1 (pPacSp1) was kindly provided by Dr R. Tjian.

**Cell culture, transient transfection and reporter gene assay**

The SV40-immortalized human fetal osteoblast cell line (SV-HFO) was a generous gift from Dr Hideki Chiba (Sapporo Medical University, Sapporo, Japan). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin G sodium (100 units/ml) and streptomycin sulfate (100 µg/ml) (Invitrogen). Cells were maintained in 5% CO₂ at 37 °C. Cells of passages 10–18 were used in the experiments described. SV-HFO cells were transfected with various promoter I.4 luciferase reporter constructs or the promoter-less luciferase vector (pGL3-Basic, Promega), along with a β-galactosidase or seapansy luciferase (phRL-TK) internal control vector (Promega), using Fugene6 transfection reagent (Roche) according to the manufacturer’s instructions. After overnight transfection, cells were treated with experimental agents for 8 h at the concentrations indicated. Firefly luciferase activity and β-galactosidase or seapansy luciferase activity were measured using the Luciferase Assay System or the Dual-Luciferase Reporter Assay System (Promega).

**Aromatase assay**

SV-HFO cells were grown to 80% confluence and then serum starved overnight. After serum starvation, cells were treated with experimental agents at the concentrations indicated. Endogenous aromatase activity in SV-HFO cells was measured by the detection of 

\[ ^3H \text{OH} \]

released during aromatization of [\( 1^\beta-^3\text{H}\) androstenedione, as previously described (Ackerman et al. 1981).

**Reverse transcriptase polymerase chain reaction (RT-PCR)**

Total RNA was prepared from SV-HFO cells using the QIAamp system (Qiagen), according to the manufacturer’s instructions. First-strand cDNA was prepared from total RNA using AMV-reverse transcriptase (Roche) and random primers (Invitrogen). PCR primer sequences for the human CYP19 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes are described by Agarwal et al. (1995). PCR conditions were as follows: 94 °C for 5 min; 1 cycle at 94 °C for 30 s, T1 °C for 30 s, 72 °C for 30 s; 10 cycles at 94 °C for 30 s, T2 °C for 30 s, 72 °C for 30 s; 10 cycles at 94 °C for 30 s, T3 °C for 30 s, 72 °C for 30 s; 10 cycles at 72 °C for 15 min; 1 cycle. Primers and temperatures were as follows: for coding region of aromatase gene transcript, RT7 and RT8 primers, T1 was 59, T2 was 57 and T3 was 55. For exon I.4-containing gene transcript, RT1 and RT8 primers, T1 was 65, T2 was 60 and T3 was 55. For exon I.3-containing gene transcript, RT3 and RT8, T1 was 60, T2 was 55 and T3 was 50. For exon I.3-containing gene transcript, RT2 and RT8, T1 was 65, T2 was 60 and T3 was 55. For human GAPDH, GAPDH1 and GAPDH2, primers were used with the following cycle conditions: 94 °C for 5 min; 1 cycle at 94 °C for 30 s, 63 °C for 30 s, 72 °C for 30 s; 25 cycles at 72 °C for 15 min; 1 cycle. Amplification of expected gene transcripts was confirmed by direct sequencing of the PCR products. RT-PCR for human GRα, STAT1 and STAT3 was performed according to published data (Sun et al. 1998, Fisher et al. 1999, Huang et al. 2001). RT-PCR for human Sp1 was performed using hSp1-S (5'–CCCTCC AGGCCTTTCAACAGCG–3') and hSp1-AS (5'-GCAGCAGTGCCCGCAGG–3') primers.

**Results**

**Aromatase activity in SV-HFO cells**

Figure 2 shows the effect of various growth factors and hormones on aromatase activity in SV-HFO cells. Basal aromatase activity was low (~0.3 pmol/mg protein per 6 h). Treatment with Dex for 24 h increased aromatase activity approximately seven-fold. Neither β-GP nor FBS had any effect on aromatase activity on its own. However, in the presence of Dex, FBS caused a further two-fold increase in aromatase activity. This suggests that a factor or factors present in serum synergized with Dex in stimulating aromatase activity. We examined a variety of hormones and cytokines for their ability to induce aromatase activity in SV-HFO (Fig. 2). Of these cytokines, only OSM was
effective. OSM had a slight stimulatory effect on aromatase activity on its own, but markedly potentiated the effect of Dex. The combination of Dex and OSM induced aromatase activity approximately 50-fold. Since aromatase activity in human adipose stromal cells can be stimulated by activators of protein kinase A (PKA) and protein kinase C (PKC) (Zhao et al. 1996), we activated these pathways by treatment with forskolin (FSK) and phorbol ester (PMA) respectively. However, these agents did not alter the aromatase activity of SV-HFO cells either alone or in combination.

Aromatase gene expression in SV-HFO cells

We next examined expression of the CYP19 gene in response to various hormones by RT-PCR, using primers designed to amplify the CYP19 coding region (Fig. 3). As a positive control, RNA from human adipose stromal cells treated with Dex and OSM, or FSK and PMA, was used. These agents induce CYP19 gene expression in adipose stromal cells from promoter I.4 (Dex + OSM) and promoter II (FSK + PMA) respectively. RT-PCR from these samples produced a band of the expected size (coding region, lanes 1 and 2). CYP19 was undetectable in SV-HFO under basal conditions (lane 3). In the presence of Dex, the CYP19 coding region was readily detected, and the level was increased upon addition of OSM, consistent with the effect of these agents on aromatase activity. This suggests that Dex and OSM act to induce aromatase activity by increasing CYP19 gene expression in SV-HFO.

The human CYP19 gene has multiple first exons derived from alternative splicing through the use of tissue-specific promoters (Fig. 1). To examine the nature of the 5' ends of the CYP19 transcripts in SV-HFO, we performed RT-PCR using exon I-specific primers. As shown in Fig. 3, CYP19 transcripts containing untranslated exon I.4 were induced in the same profile as the coding region in response to experimental treatments. Neither exon II nor the two alternative forms of exon I.3 were detected in SV-HFO under any conditions, although these were readily detectable in adipose stromal cells. Using 5'-RACE for CYP19 transcripts in SV-HFO, no sequences other than exon I.4 were detected (data not shown). Therefore, exon I.4 is predominantly selected in SV-HFO, and the
5'-promoter region of exon I.4 (promoter I.4) appears to be the only promoter used to drive CYP19 expression in these cells.

Activation of promoter I.4 in SV-HFO

To confirm direct activation of promoter I.4, a luciferase reporter construct containing 1018 nucleotides of this genomic sequence was transfected into SV-HFO (Fig. 4). Activity of this reporter gene was stimulated five- to six-fold in the presence of Dex. OSM further increased luciferase activity induced by Dex. However, no significant upregulation by β-GP, FBS or IL-1 was observed. Treatment with FSK and PMA did not affect luciferase activity, consistent with their lack of effect on aromatase activity (Fig. 2) or expression (Fig. 3).

Promoter I.4 contains a number of putative transcription factor binding sites, including sites for AP1, STAT3 (interferon γ activating sequence (GAS)), GR and Sp1. To identify the sequences that mediate induction of promoter I.4 by Dex and OSM, a series of 5'-deleted promoter I.4 constructs were transfected into SV-HFO (Fig. 5). Reporter activity in the presence of Dex and OSM decreased slightly as sequences between –1004 and –518 were deleted. Deletion of sequences between –518 and –408, which harbor the GAS element, resulted in a significant loss of reporter activity. A further significant reduction in reporter activity was observed on deletion of a 30-nucleotide sequence containing the putative GRE. Activity of the shortest construct (–244/+14), containing only the Sp1-like element, was still significantly increased in the presence of Dex and OSM compared to the empty luciferase vector (pGL3 basic), which showed no hormonal induction. These data suggest important roles for sequences surrounding the
GAS, GRE and Sp1 sites in regulating promoter I.4 in SV-HFO. We therefore mutated these putative response elements individually within the context of the full-length promoter to assess the contribution of each motif to the regulation of promoter I.4 activity.

**Role of GAS, GRE and Sp1 sites**

To assess directly the role of each element in the regulation of promoter I.4, we mutated each site individually in the context of the –1004/+14 luciferase construct (Fig. 6). As before, activity of the wild-type construct (–1004/+14) was increased three-fold and 0.5-fold in the presence of Dex and OSM respectively, and approximately five-fold by the combination of Dex (100 nM) and OSM (10%). In contrast, mutation of the GAS element (GAS mut) reduced both basal and hormone-induced reporter activity by approximately 60%, although the fold activation in the presence of stimulation was similar to that of the wild-type promoter. In contrast, mutation of the GRE reduced basal promoter activity by 70%, and abolished the response to Dex or OSM. In combination, however, Dex and OSM increased activity of the GRE mut construct four-fold. Similarly, mutation of the putative Sp1 site decreased basal promoter activity by approximately 50%, although this construct was still activated by Dex (two-fold), but not by OSM. These data confirm the importance of the GAS, GRE and Sp1 sites in regulation of *CYP19* by promoter I.4 in SV-HFO, but further suggest that no one site by itself is obligatory for transcription, since each of the mutated constructs retained some residual inducibility in response to Dex and OSM.

We next asked whether SV-HFO cells express some of the transcription factors that would be predicted to bind to these sites. Semiquantitative RT-PCR was performed using primers specific for human GRα, STAT1, STAT3 and Sp1 (Fig. 7).
Transcripts specific for each factor were detected in unstimulated cells, and there was no significant change in transcript levels of GRα, STAT1 or STAT3 on stimulation of SV-HFO cells with Dex, β-GP, OSM, FBS or FSK plus PMA. Sp1-specific transcripts, however, were slightly increased in the presence of FBS+Dex, or FSK+PMA.

**Role of GR, STAT and Sp1 proteins**

To determine whether exogenous GR, STAT or Sp1 proteins are capable of modulating transcription from promoter I.4 through these consensus sequences, SV-HFO cells were transiently transfected with the −1004/+14 construct and expression vectors encoding these transcription factors (Fig. 8). In the presence of Dex, transfection of human GRα increased promoter I.4 activity by five-fold (Fig. 8A). This effect of GRα was completely abolished when the putative GRE was mutated. Co-transfection of STAT1 had no effect on promoter I.4 activity (Fig. 8B); however, STAT3 co-transfection increased promoter activity by two-fold (Fig. 8C). STAT3 had no effect on activity of a promoter I.4 construct containing a mutation in the GAS element. Finally, transfection of Sp1 increased wild-type promoter activity four-fold, but had no effect on activity of a promoter construct containing a mutated Sp1 site (Fig. 8D). Thus, exogenous GRα, STAT3 and Sp1 can stimulate promoter I.4 activity in SV-HFO cells, in a manner dependent upon their respective *cis*-elements.
Discussion

Aromatase is expressed in a number of different tissues through the use of multiple alternative promoters. Several lines of evidence indicate that bone tissue, as well as osteoblast-like cells in primary culture, expresses aromatase and that this expression is modified in pathologic situations such as bone fracture or osteoporosis (Nawata et al. 1995, Lea et al. 1997). However, the factors that regulate aromatase expression in bone and the promoter(s) through which they act are poorly understood. Several potential regulators have been proposed, including TGF-β, 1α,25-dihydroxycholecalciferol, IL-11, TNFα, vitamin D and 17β-estradiol (Tanaka et al. 1996, Shozu & Simpson 1998, Shozu et al. 2000, Enjuanes et al. 2003). Although several different promoters have been implicated in the action of these hormones, the primary promoter utilized appears to be promoter I.4, with relatively minor contributions from promoters II, I.3, I.2 and I.6 (Shozu & Simpson 1998, Enjuanes et al. 2003). The recent development of the human osteoblast-derived SV-HFO cell line (Chiba et al. 1993) provides a valuable model in which to dissect the mechanisms regulating aromatase expression in osteoblasts. SV-HFO cells exhibit osteoblastic markers such as alkaline phosphatase and calcitonin, respond to the osteotropic factors vitamin D3 and retinoic acid, and undergo mineralization in response to glucocorticoids.

In the present study we found that aromatase activity and gene transcript level in SV-HFO cells were stimulated by Dex in SV-HFO cells. This is consistent with previous studies in primary human osteoblast-like cells and THP-1 monocytic cells (Tanaka et al. 1996, Shozu et al. 2000, Takayanagi et al. 2002). Although glucocorticoid excess is well

Figure 6 Effect of mutation of putative CYP19 promoter I.4 cis-elements. –1004/+14 Luc and the indicated mutants were transfected into SV-HFO and stimulated with Dex (100 nM) and OSM (5 ng/ml), either alone or in combination. Gray symbols represent the mutated elements. Experiments were performed in triplicate, and similar results were obtained in at least two further experiments. Error bars indicate standard error.
known to induce osteoporosis – mainly through inhibition of intestinal calcium absorption – physiological concentrations of glucocorticoids can produce osteoplastic effects (Canalis 1983). Thus, the induction of aromatase expression in osteoblasts by low concentrations of glucocorticoids, as shown here, is not inconsistent with their osteoporotic effects at high concentrations.

Of the other agents tested in this study, OSM was the only factor found to induce aromatase activity on its own, and this effect was markedly enhanced by Dex. In this regard, mice with targeted gene disruption and transgenic mice have revealed an important relationship between gp130 cytokines and bone formation (Heymann & Rousselle 2000). Metallothionein promoter-driven bovine oncostatin M gene expression caused excessive growth of bone in the femur and phalanges (Malik et al. 1995). This suggests a potent positive effect of OSM on bone formation and probably on maturation of osteoblasts. From the results obtained here, these actions of OSM could be related, in part, to a stimulation of aromatase expression. Further analysis of the effects of these agents on alkaline phosphatase activity, osteocalcin expression or mineral deposition should provide insight into this relationship.

The dominant promoter used to express aromatase in SV-HFO was found to be promoter I.4 and, consistent with the lack of effect of the PKA pathway or PKC pathway upon aromatase activity, promoter II was not utilized in SV-HFO (Fig. 3). These data suggest that the profile of aromatase regulation in SV-HFO is similar to that of primary human osteoblasts (Shozu & Simpson 1998). SV-HFO is therefore a relevant and useful model with which to investigate aromatase regulation in osteoblasts. In human adipose stromal cells, CYP19 gene transcripts are increased in response to either Dex + OSM or FSK + PMA; DEX + OSM induces CYP19 expression via promoter I.4, whereas FSK + PMA induces expression via promoter II (Zhao et al. 1995a,b). Promoter II was not activated in SV-HFO cells under any of the experimental conditions, and this is one important difference between aromatase expression in adipose stromal cells and osteoblasts.
Figure 8  GRα, STAT3 and Sp1 induce promoter l.4 reporter activity in SV-HFO. SV-HFO cells were co-transfected with −1004/+14 Luc, or derivatives thereof containing mutations in the indicated cis-elements (GRE, GAS or Sp1), and expression vectors encoding (panel A) GRα; (panel B) STAT1; (panel C) STAT3 or (panel D) Sp1. The total amount of DNA was normalized to 1.5 µg with the parent pCNDNA3.1+ vector. Cells were then stimulated with Dex (100 nM) and OSM (5 ng/ml) for 8 h before being lysed and assayed for luciferase activity. Data represent the mean±S.E.M. of three independent experiments, and are expressed relative to control activity (100%).
The results of promoter deletion/mutation experiments suggest important roles for the GAS, GRE and Sp1 sites in promoter I.4 regulation in SV-HFO. In adipose stromal cells, activators of gp130-coupled receptors, such as OSM, and glucocorticoids induce promoter I.4 through these same elements. The action of OSM is associated with phosphorylation and activation of homo- or heterodimers of STAT1 and STAT3, which bind to the GAS element to induce specific genes (Gomez-Lechon 1999). Induction of promoter I.4 activity via binding of STAT3 to the GAS element in response to IL-11 in adipose stromal cells has been reported (Zhao et al. 1995b). Glucocorticoids, on the other hand, stimulate CYP19 transcription through a classical GR-GRE-mediated pathway in adipose stromal cells (Zhao et al. 1995a). Although we have not yet identified the transcription factors that bind the GAS element and GRE in SV-HFO, transfection of GRα, STAT3 or Sp1 increased promoter I.4 activity, and RT-PCR analysis indicated that these transcription factors are expressed in SV-HFO cells.

While it is therefore likely that the transcriptional mechanisms used to drive promoter I.4 in SV-HFO and adipose stromal cells are similar, the results of the present study have revealed subtle differences between the two cell types. In the first place, as mentioned above, promoter II is not activated in SV-HFO cells, in contrast to adipose stromal cells. Secondly, in adipose stromal cells, promoter I.4 has an obligatory requirement for glucocorticoids, although glucocorticoids by themselves have no effect on aromatase activity. In contrast, glucocorticoids alone increase aromatase activity and gene expression in SV-HFO cells. Lastly, a number of class I cytokines activate promoter I.4 in adipose stromal cells, whereas in SV-HFO cells, only OSM is effective. These differences presumably reflect divergent mechanisms that dictate cell fate from mesenchymal stem cells.

From a clinical standpoint, recognition that aromatase activity within bone cells is the major source of estrogen influencing this tissue leads to several considerations. First, it suggests that circulating androgens rather than estrogen are important precursors of estrogen within bone, since circulating testosterone levels in blood of postmenopausal women are an order of magnitude greater than these in postmenopausal women, and this high level of estrogen precursor could be one reason why men are less at risk of bone loss than are women. Finally, aromatase inhibitors are widely used for the treatment of breast cancer patients. However, these inhibitors inhibit aromatase in a global fashion, including bone, and this could lead to loss of bone mineralization. Although this might be of less consequence for elderly women with advanced postmenopausal breast cancer, the effects of estrogen deprivation in bone of young postmenopausal women could be significant. This is particularly relevant given the current interest in the use of aromatase inhibitors as preventative therapies. To avoid this kind of risk, the development of selective aromatase modulators (SAMs) that inhibit aromatase activity only in breast tissue, but not in bone tissue, would be useful (Simpson et al. 2000, Simpson & Davis 2001). The concept of SAMs is based on the fact that (a) local aromatase activity is the main source of estrogen in breast and bone, and (b) the mechanism of aromatase expression is different between breast adipose stromal cells and osteoblasts (for example, promoter II is activated in adipose stromal tissues of breast cancer patients, but not in osteoblasts). Thus, inhibition of promoter II-mediated transcription as breast cancer therapy (Clyne et al. 2002) would have little effect on local estrogen production in bone.

**Acknowledgements**

We thank Dr Hideki Chiba, Sapporo Medical University (Sapporo, Japan), for his kind gift of the SV-HFO cell line. We thank Mr Fumihiko Yukinawa for his technical assistance. This work was supported by a grant from the Victorian Breast Cancer Research Consortium and by USPHS Grant no. R-37 AG-08174.

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Received in final form 27 October 2003

Accepted 3 December 2003