Comparison of Human and Rat Uterine Leiomyomata: Identification of a Dysregulated Mammalian Target of Rapamycin Pathway


Abstract

Uterine leiomyomata, or fibroids, are benign tumors of the uterine myometrium that significantly affect up to 30% of reproductive-age women. Despite being the primary cause of hysterectomy in the United States, accounting for up to 200,000 procedures annually, the etiology of leiomyoma remains largely unknown. As a basis for understanding leiomyoma pathogenesis and identifying targets for pharmacotherapy, we conducted transcriptional profiling of leiomyoma and unaffected myometrium from humans and Eker rats, the best characterized preclinical model of leiomyomata. A global comparison of mRNA from leiomyoma versus myometrium in human and rat identified a highly significant overlap of dysregulated gene expression in leiomyomata. An unbiased pathway analysis using a method of gene-set enrichment based on the sigPathway algorithm detected the mammalian target of rapamycin (mTOR) pathway as one of the most highly up-regulated pathways in both human and rat tumors. To validate this pathway as a therapeutic target for uterine leiomyomata, preclinical studies were conducted in Eker rats. These rats develop uterine leiomyomata as a consequence of loss of Tsc2 function and up-regulation of mTOR signaling. Inhibition of mTOR in female Eker rats with the rapamycin analogue WAY-129327 for 2 weeks decreased mTOR signaling and cell proliferation in tumors, and treatment for 4 months significantly decreased tumor incidence, multiplicity, and size. These results identify dysregulated mTOR signaling as a component of leiomyoma etiology across species and directly show the dependence of uterine leiomyomata with activated mTOR on this signaling pathway for growth. [Cancer Res 2009;69(15):6171–8]

Introduction

Uterine leiomyomata, or fibroids, are benign tumors of the uterine myometrium and occur in up to 77% of reproductive-age women (1). Clinically significant leiomyomata occur in 30% of these women (1), resulting in a spectrum of gynecologic symptoms, including menorrhagia/anemia, pelvic pain/pressure, dyspareunia, and, in some cases, reproductive dysfunction. Leiomyomata are the primary cause of hysterectomy in the United States, accounting for up to 200,000 procedures annually.

Despite its prevalence, the pathogenesis of uterine leiomyomata is unknown. It has been suggested that leiomyomata grow under the control of ovarian steroids and their receptors and growth factors (such as epidermal growth factor, insulin growth factor, and their binding proteins, and transforming growth factor-β; ref. 2). Nonetheless, the factors that promote both the initial development and subsequent in vivo growth remain poorly understood. Toward the goal of understanding the pathogenesis of leiomyomata, multiple studies have been performed to identify genes that are dysregulated in leiomyoma versus normal myometrium. Compilation of all such microarray data has led to the identification of genes involved in broad pathways, such as retinoid metabolism, growth and proliferation, differentiation, and extracellular matrix maintenance (3).

The limited number of animal models for this disease has also hindered the advancement of leiomyoma research. Models have been published in several species, including mouse (4–6), rat (7), guinea pig (8–10), and potbellied pig (11). The Eker rat, first described in 1954 as a rodent model of renal cell carcinoma, was subsequently observed to develop spontaneous uterine leiomyomata in ~65% of female rats by 16 months of age (12, 13). The predisposing genetic alteration was identified as an endogenous retroviral insertion in the Tsc-2 tumor suppressor gene (14, 15). Consistent with Knudson’s two-hit hypothesis of tumorigenesis, leiomyomata develop in rats heterozygous for the Eker mutation (Tsc2F1/+), through spontaneous inactivation of the remaining wild-type allele (7). Eker rat leiomyomata share phenotypic, biochemical, and genetic characteristics with the cognate human disease, including estrogen and progesterone receptor expression, responsiveness to steroid hormones, aberrant HMGA2 expression, overexpresion of insulin-like growth factor-I, and the protective effects of pregnancy, making it potentially the best rodent model of leiomyoma.

Here, we report the most extensive microarray study to date using matched human leiomyoma versus myometrium from 23 patients and the first cross-species microarray analysis of rat leiomyoma versus myometrium. Comparison of the two species-specific data sets and bioinformatic analysis of these microarray data identified the mammalian target of rapamycin (mTOR) pathway as one of the most highly up-regulated pathways in leiomyomata from both species. Because mTOR is central to the ability of a cell to integrate external signals such as growth factors, nutritional status, and stress to direct proliferation (16, 17), the discovery that this pathway is dysregulated in leiomyomata was both provocative and sensible. In vivo and in vitro studies confirmed

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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activation of the mTOR signaling pathway in leiomyomata, and treatment of Eker rats with the rapamycin analogue WAY-129327 inhibited mTOR signaling and decreased tumor incidence, multiplicity, and size. These data show the important role of mTOR signaling in leiomyoma etiology and the potential pharmacotherapeutic opportunities for targeting this pathway in the treatment of this disease.

Materials and Methods

Rat tissue collection. The care and handling of rats was in accordance with NIH guidelines and conducted in Association for the Assessment and Accreditation of Laboratory Animal Care–accredited facilities. All animal studies were approved by the Institutional Animal Care and Use Committee of the M.D. Anderson Cancer Center. Fifteen leiomyoma and 16 myometrium samples were collected from 12- to 17-mo-old female Eker rats (Long Evans; Tsc-2/Ek/+), including four matched tumor/normal pairs. Samples were immediately frozen at −80°C until RNA was isolated.

Human clinical study design. All human studies were performed according to the principles of the Declaration of Helsinki and were approved by the Institutional Review Board/Independent Ethics Committees of Kuopio University Hospital, Kuopio, Finland; Meharry Medical College, Nashville, TN; and Quorum Review, Inc., Seattle, WA. This multicenter, nontreatment, clinical study was initiated to collect uterine leiomyoma and normal myometrial biopsy specimens from premenopausal patients undergoing standard-of-care hysterectomy for uterine leiomyomata. Written informed consent was obtained from all subjects before any study-related procedure.

Twenty-three women were enrolled in the study. Mean demographic data are presented in Supplementary Table S1. Study eligibility of each subject was confirmed by medical and reproductive history, vital signs, medication use, a physical and gynecologic examination including pap smear, and various laboratory assessments including hematology, serum chemistry, HbA1c, serum estradiol, and serum follicle-stimulating hormone levels. Following confirmation of subject eligibility, up to three specimens (1 cm³ each) of leiomyoma and normal myometrium were collected from each subject at the time of hysterectomy. Information on location of tumor, size of tumor, and menstrual phase were noted at the time of sample collection (Supplementary Table S2). Samples were immediately frozen at −80°C until RNA was isolated.

RNA preparation and hybridization. RNA extraction, isolation, and labeling was performed as previously described (18). For rat samples, 10 μg of cRNA were fragmented and hybridized to GeneChip Rat Genome 230
2.0 arrays according to the manufacturer's protocol (Affymetrix). For human biopsies, 10 μg cRNA were fragmented and hybridized to GeneChip Human Genome U133 Plus 2.0 arrays. Raw data can be found in the GEO database as accession GSE13319.7

Differentially expressed probe sets. All analysis methods are detailed in the Supplementary Methods. Briefly, loess normalized signal values were tested for differential expression between leiomyoma and myometrium using CyberT (19) for the rat data set, and Bioconductor's LIMMA package (20) for the human data set. Using a false-discovery rate (FDR) cutoff of 0.001, 5,834 and 2,222 probe sets were determined to be significantly differentially expressed in rat and human, respectively. The range of significant fold changes was 0.014 to 78.5 for rat and 0.08 to 22.3 in human.

Determination of homologous qualifiers. Affymetrix probe set annotations8 were used to assign Entrez Gene IDs to each probe set, Bayesian t statistics for probe sets querying the same gene were averaged together to obtain gene-level t statistics and FDR values (Supplementary Table S3). To compare between species, 11,263 orthologous rat-human gene pairs were obtained using National Center for Biotechnology Information's (NCBI) Homologene (build 63; ref. 21; see Supplementary Methods for details).

Identification of significantly regulated gene sets. Significantly regulated biological pathways were identified using an internal R implementation of the sigPathway algorithm (22) incorporating specific modifications as detailed in the Supplementary Methods. Selected gene sets from the Broad Institute's MSigDB collection (23) were tested. A gene set was considered significant when FDR(NEk) ≤ 0.01 and FDR(NEk) ≤ 0.01 for each of the human and rat leiomyoma versus normal comparisons. Eighty-eight gene sets passed these cutoffs (Supplementary Table S4) and were called differentially expressed in both human and rat.

Assessment of mTOR dysregulation in individual fibroid samples. A method was devised to estimate the fraction of human leiomyoma samples with mTOR dysregulation from the transcriptional profiling data set. For each leiomyoma sample, we calculated the fraction of rapamycin–downregulated probe sets with higher expression in that leiomyoma relative to the donor-matched myometrium sample. The distribution of these fraction values over all leiomyomata was then compared with values generated from random gene sets (see Supplementary Methods).

Antibodies and Western blot analysis. Western blot analysis was performed with phospho-S6 (serine235/236) S6 ribosomal protein (Cell Signaling Technology) or anti-Ki67 (DAKO) according to the manufacturer’s instructions. Sections were count-stained with hematoxylin, dehydrated, and mounted. Controls that lacked primary antibody were incubated in 1× PBS with 10% goat serum in each experiment. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed with the FragEL DNA Fragmentation Detection kit (Calbiochem) per the manufacturer’s instructions. Ki67 and TUNEL labeling indices were scored by assessing the number of cells positive for Ki67 nuclear antigen or TUNEL/100 cells counted.

Results

Identification of genes dysregulated in uterine leiomyoma tissue. Differential expression of genes between leiomyoma and uninvolved myometrial tissue was determined in human clinical samples and in the Eker rat model of leiomyomata. Analysis was restricted to 11,263 genes that had orthologous sequences on the arrays from both species. We identified 1,543 regulated genes in human samples (FDR ≤ 0.01; Fig. 1) of which 1,069 showed a similar direction of change in the rat (578 of these passed FDR ≤ 0.01). Comparing the t statistics of differential gene expression between

Figure 2. mTOR pathway dysregulation in human and rat. A, heat map shows the leiomyomata versus myometrium gene-level t statistics values for the Peng and colleagues Rap_down gene set (25) in the human and rat microarray data. The abundance of red indicates that this gene set is up-regulated in both human and rat leiomyomata. Red, leiomyoma > myometrium; blue, leiomyoma < myometrium. B, quantitation of mTOR pathway up-regulation in individual human leiomyoma tumors using the Peng and colleagues Rap_down gene set (25). The X axis, “fraction up-regulated,” is the fraction of probe sets in the Rap_down gene set that are up-regulated in each individual tumor sample. Red, the distribution of this fraction up-regulated value over all leiomyomata. Solid black line, distribution obtained from random gene sets using the same human microarray data. Dotted black line, cutoff corresponding to a FDR of 0.01. Individual tumors plotted to the right of the FDR cutoff are predicted to have up-regulated mTOR signaling (39 of 52 tumors or 75%).

2 http://www.affymetrix.com/analysis/downloads/na27/retv/
leiomyomata and myometrium in human versus rat resulted in a positive Pearson correlation coefficient of 23% (Fig. 1B). Although seemingly small in magnitude, this correlation coefficient is highly statistically significant, indicating overall positive concordance between the two data sets. Included in the genes that show similar regulation in both species are glutamate ionotropic receptor 2, calpain 6, matrix metalloproteinase 11, and matrix metalloproteinase 13 (Supplementary Fig. S1).

**Human-rat pathway analysis.** We identified dysregulated gene sets in human and rat leiomyomata using a method of gene set enrichment (22) and an a priori defined set of genes derived from the Broad Institute's MSigDB collection (23). NTk and NEk scores were independently calculated for both human and rat data. We identified 88 gene sets that were significantly regulated (FDR < 0.01) in both the rat and human, with all 88 showing concordant direction of change between human and rat. A comparison of the NTk scores generated from human and rat showed a Pearson correlation of 55% (Fig. 1C). Consistent with what is known about the etiology of uterine leiomyomata, our analysis of dysregulated gene sets indicated that in both species, altered cell cycle regulation, increased extracellular matrix production, and altered amino acid metabolism were characteristics of these tumors.

One of the most highly up-regulated gene sets (overall rank = 13) in both the rat and human included a set of genes that is down-regulated following treatment with the mTOR inhibitor rapamycin (ref. 25; Fig. 2A), suggesting that altered mTOR signaling was a feature of uterine leiomyomata. Of the genes in this gene set satisfying a FDR ≤ 0.1 cutoff for differential expression, 86% of them were up-regulated. Based on the human microarray data, we estimated the percentage of human leiomyomata with increased mTOR signaling at the transcriptional level by analyzing the human microarray data using two independent data sets that identified rapamycin-down-regulated genes (Rap_down gene sets; refs. 25, 26).

**Inhibition of mTOR signaling by WAY-129327.** To test the hypothesis that mTOR signaling contributed to the growth of uterine leiomyomata, we conducted an initial set of experiments in vitro using the rapamycin-related mTOR inhibitor WAY-129327 (see Fig. 3A for structure) and a rat leiomyoma–derived cell line, ELT3 (24). Treatment of ELT3 cells with 10, 50, or 100 nmol/L WAY-129327 inhibited phosphorylation of the downstream mTOR target S6K and its effector S6 and resulted in a dose-dependent decrease (IC50 6.8 ± 0.92 nmol/L, n = 14) in cell proliferation (Fig. 3B and C).

To evaluate mTOR signaling as a potential therapeutic target, efficacy of WAY-129327 inhibition of mTOR signaling (at 2 weeks and 4 months) and impact on growth uterine leiomyomata in vivo (at 4 months) was determined. Fifteen-month-old Eker rats were treated with WAY-129327 for 2 weeks to evaluate target engagement (i.e., inhibition of mTOR signaling). Immunohistochemical staining for pS6 confirmed activation of mTOR signaling in vehicle-treated Eker rat leiomyomatia, and treatment with WAY-129327 resulted in complete inhibition of mTOR signaling in these tumors (Fig. 4). Whereas 100% (16 of 16) of the vehicle tumors were positive for phospho-S6, 0% (0 of 13) of the WAY-129327–treated tumors exhibited phospho-S6 immunoreactivity (P < 0.001, χ² analysis; Supplementary Table S5). This result was confirmed by Western analysis of tumors, which showed decreased phosphorylation of S6 and S6K in WAY-129327–treated tumors, with no change in total levels of these proteins (Fig. 5 and data not shown). 4EBP-1 phosphorylation, however, was not decreased, consistent with published results that this phosphorylation may have differential sensitivity to rapamycin analogue treatment (refs. 27,
Inhibition of mTOR signaling correlated with decreased cell proliferation at this 2-week time point, with the Ki67 proliferative index decreasing from 21% to 2.8% in vehicle versus WAY-129327–treated cells, respectively, although no change was observed in apoptotic index as measured by TUNEL staining (Supplementary Table S5).

The ability of WAY-129327 to block mTOR signaling and inhibit cell proliferation in leiomyoma cells in vitro and in vivo suggested that this mTOR inhibitor could be efficacious for treatment of these tumors. To test this experimentally, Eker rats were treated with WAY-129327 or vehicle for 4 months. As shown in Table 1, the tumor incidence was significantly reduced from 76.6% (36 of 47) in vehicle-treated rats to 38.6% (17 of 44; \( P < 0.0001 \) Fisher’s exact test) with WAY-129327 treatment. An impact was also observed on large tumors, with the incidence of grossly observable tumors decreasing from 34% (16 of 47) to 11% (5 of 44; \( P = 0.056 \) Fisher’s exact test) with WAY-129327 treatment. Additionally, tumor multiplicity was also significantly decreased from 1.41 to 0.55 tumors per rat (\( P < 0.001 \) t-test) and tumor size, which for grossly observable tumors was reduced from an average of 3.9 cm\(^3\) in vehicle-treated animals to 3.1 cm\(^3\) in animals treated with WAY-129327 (\( P < 0.001 \) nonparametric Wilcoxon two-sample test). As with the 2-week treatment, the 4-month apoptotic indices were unchanged, whereas the proliferative index of leiomyoma cells decreased from 22.4% to 3.2% (\( P < 0.01 \) t-test; Supplementary Table S5).

**Discussion**

Despite the prevalence of uterine leiomyomata, the pathogenesis of this tumor type remains unclear. Attempts to understand uterine leiomyomata etiology have included epidemiologic (29), cytogenetic, and microarray investigations of human leiomyomata versus unaffected uterine myometrium. Cytogenetic studies led to the identification of common chromosomal rearrangements involving hormone-regulated gene products such as fumarate hydratase, aromatase, and the high mobility group proteins (30). However, chromosomal rearrangements are not consistently found in all tumors and no firm link has been established between these hormone-regulated genes and uterine leiomyoma tumorigenesis. Investigations into the role of steroid hormones have led to a better, albeit incomplete, understanding of the role of estrogen and progesterone in leiomyoma pathogenesis and to the development of...
Transforming growth factor receptor, ionotropic AMPA2 (binding protein) (CDKN1A) and the transforming growth factor receptor II (TGFBR2) are also differentially expressed in our comparing microarray studies, many genes reported to be dysregulated systems/pathways at work in these tumors. A global comparison of mRNA from leiomyoma versus myometrium in human versus rat resulted in a highly significant overlap of regulated genes (correlation of 0.23). Our estimation of the correlation is likely to be lower than actual, because some probe sets on the arrays could be potentially be mistargeted, nonfunctional, or target alternative splice forms. Although the correlation may seem low, it is consistent with previously published cross-species analyses. The t statistic correlation for genes expressed in estradiol-treated vaginal tissue from human and rat is reported to be 0.33 (34) and McC Carroll and colleagues (35) determined a t statistic correlation of 0.15 in the transcriptional response to aging in flies versus worms, which they note to be highly significant. Thus, an ~ 23% correlation coefficient is within the expected value range.

Single gene analyses have been conducted previously in uterine leiomyomata and, although highly significant and suggestive, are unable to capture all tumor-associated changes in biological pathways. For cross-species comparisons, gene-by-gene comparisons have been used to highlight similarities in disease models based on the principles that important biological processes are more likely to be conserved, but these comparisons use relatively small data sets compared with the vast amount of data generated by global analysis. Furthermore, comparisons across species can be complicated by differences in regulatory mechanisms, a problem that can be circumvented by grouping genes into sets consisting of predetermined functional or related categories and identifying trends within a given gene set. Here, an unbiased pathway approach was performed for human and rat uterine leiomyomata using high-content data sets to identified pathways dysregulated in leiomyomata of both species, demonstrating the power of cross-species comparisons. For example, genes involved in cell signaling and control of the cell cycle pathways such as TGFBR3 and CDKN1A are up-regulated in leiomyomata from both species, as are extracellular matrix component genes such as the collagens [collagen, type IV (COL4A1, COL4A2), type V (COL5A1), and type VI (COL6A1)] and some matrix metalloproteinases (MMP10, MMP7, MMP11, and MMP14). Wnt signaling pathway genes including WNT inhibitory factor (WIF1), WNT1 inducible signaling pathway protein 1 (WISP1), and dickkopf homologue 2 (DKK2) are also up-regulated in both species. In the retinoic acid pathway, ALDH1A1, ALDH1A2, CRABP1/2, and ADH1 were also aberrantly expressed.

Table 1. Tumor burden in Eker rats treated for 4 months with WAY-129327 compared with vehicle

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Multiplicity</th>
<th>Size (cm²)</th>
<th>Incidence (%)</th>
<th>Gross</th>
<th>Overall</th>
<th>Gross tumors</th>
<th>Microscopic tumors</th>
<th>No tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No. rats with lesions</td>
<td>Total no. lesions</td>
<td>No. rats with lesions</td>
</tr>
<tr>
<td>VEH (n = 47)</td>
<td>1.41</td>
<td>3.9</td>
<td>34</td>
<td>77</td>
<td>16</td>
<td>19</td>
<td>27</td>
<td>51</td>
</tr>
<tr>
<td>WAY (n = 44)</td>
<td>0.51</td>
<td>3.1</td>
<td>11</td>
<td>39</td>
<td>5</td>
<td>5</td>
<td>14</td>
<td>19</td>
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</table>

Note: See text for statistical analysis.
Abbreviations: VEH, vehicle; WAY, WAY-129327.
One pathway of particular interest that was identified by this approach is the mTOR pathway. As noted earlier, mTOR is a downstream protein kinase in the phosphatidylinositol 3'-kinase/akt pathway and is a central modulator of many cell processes (including cell proliferation) in response to various cellular stimuli (as reviewed in refs. 16, 17). Dysregulation of this pathway has been implicated in a variety of pathologic conditions including metabolic syndrome, cancer, autoimmune disorders, cardiac hypertrophy, neurologic disorders, and more recently, benign proliferative disorders such as lymphaangiomyomatosis, tuberous sclerosis complex syndrome, and uterine leiomyomata. We compared our expression data to two independent data sets that identified genes down-regulated by rapamycin in B-lymphoma cells or in Tsc-2 null mouse embryo fibroblasts (25, 26). These two studies represent diverse biological backgrounds yet significantly correlated with the mTOR signature seen in uterine leiomyomata, suggesting that the effects of mTOR activation are a consistent feature associated with dysregulation of this signaling pathway.

Tumors in the Eker rat arise due to inactivation of the Tsc-2 tumor suppressor, leading to unregulated mTOR signaling and cell proliferation. As a result, Eker rats are predisposed to develop renal cell carcinoma, splenic hemangiosarcoma, pituitary adenoma, and uterine leiomyomata. In previously published work, inhibition of the mTOR pathway by rapamycin reduced kidney tumor volume and reversed symptoms of pituitary adenoma in the Eker rat (36), demonstrating the dependence of these tumors on mTOR signaling. Additionally, the data obtained here using the Eker rat are consistent with human data obtained in other settings of mTOR dysregulation. For example, treatment with rapamycin decreases restenosis following angioplasty in vascular smooth muscle (37), inhibits proliferation of estrogen receptor–positive breast cancer (38), and reverses estrogen-induced proliferation of uterine leiomyoma and myometrium-derived cell lines (39). However, targeting mTOR as a therapeutic strategy for uterine leiomyomata has not previously been examined in the clinic or in animal models.

Our determination that the mTOR pathway was up-regulated in human leiomyomata was based on a global transcriptional analysis that identified multiple rapamycin-sensitive genes as being up-regulated in these tumors. Overall, this profile was observed in ~75% of the human tumors examined. We attempted to confirm this pathway analysis at the protein level (pS6 phosphorylation using Western analysis) but results were inconclusive (data not shown). S6 phosphorylation was increased in about a third of tumors tested, with the remainder evenly split between decreased or no change in phosphorylation. These apparently discordant results may be explained by several possibilities. First, from a technical standpoint, it is difficult to efficiently extract proteins from leiomyoma tissue because a large proportion of the tumor is often highly fibrous and acellular. Second, a pathway analysis is a more sensitive and powerful tool than assessment of a single end point, as it measures multiple end points across many pathways. As such, many changes, even small but in concordant direction, register as significant, whereas Western analysis measures a specific end point using a method that is less precisely quantifiable. Third, many leiomyomata grow slowly and may be expected to have a different pS6 profile than faster growing malignant tumors. Clearly, given the positive preclinical data obtained in this study with WAY-129327, the best test of whether an mTOR inhibitor can benefit human patients suffering from leiomyomata will be a randomized, double-blind, placebo-controlled clinical trial, ideally one in which mTOR activation can be incorporated into patient selection and/or used as a biomarker of efficacy. As with any human study, a universal response is not expected. In human, a diversity of risk factors contributing to leiomyomata growth have been identified, including genetic alterations, obesity, nulliparity, and race (2). Our expression profiling data predict that in the majority of tumors, these independent dysregulated pathways ultimately affect the mTOR signaling cascade.

In conclusion, the current study has identified a wealth of changes that occur at the molecular level in uterine leiomyomata in both the human and Eker rat. These data lend additional confidence that the Eker rat is a reasonable model of the human disease, a finding with the potential to facilitate future drug discovery efforts. Importantly, our pathway-driven analysis of microarray data, identification of dysregulated mTOR signaling in uterine leiomyomata, and efficacy shown in the Eker rat with the mTOR inhibitor WAY-129327 suggest that mTOR inhibitors may hold promise as a medicinal therapy for this disease.

Disclosure of Potential Conflicts of Interest

J.S. Crabtree, S.A. Jelinsky, H.A. Harris, S.E. Choe, M.M. Cotreau, M.L. Kimberland, E. Wilson, K.A. Saraf, V. Jiu, E.L. Brooks, W. Kao, J.S. Skottnicki, M. Abou-Ghazia, and R.C. Winneker were full-time employees of Wyeth Research at the time this work was conducted. The authors disclosed no potential conflicts of interest.

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