Genome-Wide DNA Methylation Indicates Silencing of Tumor Suppressor Genes in Uterine Leiomyoma

Antonia Navarro¹, Ping Yin¹, Diana Monsivais¹, Simon M. Lin², Pan Du², Jian-Jun Wei³, Serdar E. Bulun¹

¹ Division of Reproductive Biology Research, Department of Obstetrics and Gynecology, Feinberg School of Medicine, Northwestern University, Chicago, Illinois, United States of America, ² Bioinformatics Core, Feinberg School of Medicine, Northwestern University, Chicago, Illinois, United States of America, ³ Department of Pathology, Feinberg School of Medicine, Northwestern University, Chicago, Illinois, United States of America

Abstract

Background: Uterine leiomyomas, or fibroids, represent the most common benign tumor of the female reproductive tract. These tumors become symptomatic in 30% of all women and up to 70% of African American women of reproductive age. Understanding the molecular basis for tumor development and progression, particularly those associated with gene silencing, is urgently needed. Epigenetic dysregulation of individual genes has been demonstrated in leiomyoma cells; however, the in vivo genome-wide distribution of such epigenetic abnormalities remains unknown.

Principal Findings: We characterized and compared genome-wide DNA methylation and mRNA expression profiles in uterine leiomyoma and matched normal myometrial tissues from 18 African American women. We found 55 genes with differential promoter methylation and concomitant differences in mRNA expression in uterine leiomyoma versus normal myometrium. Eighty percent of the identified genes showed an inverse relationship between DNA methylation status and mRNA expression in uterine leiomyoma tissues, and the majority of genes (62%) displayed hypermethylation associated with gene silencing. We selected three genes, the known tumor suppressors KLF11, DLEC1, and KRT19 and verified promoter hypermethylation, mRNA repression and protein expression using bisulfite sequencing, real-time PCR and western blot. Incubation of primary leiomyoma smooth muscle cells with a DNA methyltransferase inhibitor restored KLF11, DLEC1 and KRT19 mRNA levels.

Conclusions: These results suggest a possible functional role of promoter DNA methylation-mediated gene silencing in the pathogenesis of uterine leiomyoma in African American women.

Introduction

Uterine leiomyomas or fibroids are benign smooth muscle tumors of myometrial origin; despite their benign nature, they are able to undergo rapid and considerable growth [1]. Uterine leiomyomas are the most common gynecological tumors in women of reproductive age, and they become symptomatic in 25–30% of all women and in up to 70% of African American women of reproductive age [2]. Compared with white women, African American women are 3 times more likely to develop symptomatic leiomyoma, which also develops at earlier ages with more numerous and larger fibroids [3]. The clinical symptoms associated with uterine leiomyomas are abnormal uterine bleeding, which can lead to anemia, pelvic pressure and pain; reduced fertility; and frequent pregnancy loss [4]. In the United States, 600,000 hysterectomies are performed each year; of these, approximately 40% are performed to treat uterine leiomyoma. The surgical costs alone represent an economic burden of $2 billion per year [5], and when taking into account the social costs and associated long-term health problems, it is clear that better prevention and treatment options for women with uterine leiomyoma are urgently needed. Understanding the molecular mechanisms underlying the pathogenesis of uterine leiomyoma will facilitate the discovery and development of new approaches to the treatment of this disease.

Gene expression profile studies have demonstrated that hundreds of genes with critical functions in differentiation, apoptosis, proliferation and extracellular matrix formation are dysregulated in uterine leiomyoma [6]. Currently, a few cytogenetic aberrations in specific genes have been discovered [7]; however, it remains unknown whether these dysregulated genes act as effectors or growth promoters in uterine leiomyoma. Epigenetic mechanisms such as DNA methylation, histone modification, and non-coding RNAs are described as heritable changes in gene expression not associated with changes in the primary DNA sequence; rather, these changes affect secondary interactions that play a crucial role in the regulation of gene expression. In the mammalian genome, DNA methylation is the most common and well-characterized epigenetic mark, which consists of the covalent addition of a methyl group to the 5’-carbon of the cytosine ring within the context of CpG dinucleotides following replication. The methylation of this cytosine is catalyzed by specific DNA methyltransferases (DNMTs), which transfer a methyl group, from the donor S-adenosyl methionine (SAMe) to
the 5'-position of the pyrimidinic ring [8]. Recent studies reveal that there is differential expression of DNMTs in uterine leiomyoma and that there is aberrant DNA methylation in uterine leiomyoma compared with normal myometrial tissue [9–10]. One study demonstrated that hypomethylation of ESR1 in uterine leiomyoma correlates with increased mRNA expression in uterine leiomyoma [11]. These findings suggest that, DNA methylation might play a key role in the pathogenesis of uterine leiomyoma by altering the normal myometrial mRNA expression profile. Further characterization of the role of epigenetics in the tumorigenesis of uterine leiomyoma, requires an analysis of global, genome-wide DNA methylation in disease and normal uterine tissue.

The objective of this study was to determine the relationship between differential DNA methylation and mRNA expression in uterine leiomyoma by performing a genome-wide analysis. We sought to determine whether differentially regulated genes in uterine leiomyoma versus adjacent normal myometrial tissue are under epigenetic control. We attempted to identify a subset of genes whose differential DNA methylation correlated with differential mRNA expression. Our findings will advance our understanding of the contribution of DNA methylation to the pathogenesis of uterine leiomyoma.

**Results**

**Analysis of DNA methylation and mRNA expression in uterine leiomyoma and matched adjacent myometrial tissue**

We compared DNA methylation patterns in human uterine leiomyoma and adjacent normal myometrial tissues from 18 women to identify genes that are differentially expressed and epigenetically regulated. We used samples from African American women to limit biological heterogeneity and avoid epigenetic variation among ethnic groups; key clinical characteristics of samples are described in Table 1. We performed a genome-wide DNA methylation analysis using the high throughput Illumina Infinium Human Methylation27 Beadchips in paired sets of uterine leiomyoma and matched adjacent normal myometrial tissues. The Illumina probes detect the methylation state of 27,578 individual CpG dinucleotides, located predominantly in CpG islands within proximal promoter regions between 1.5 kb upstream and 1 kb downstream of the transcription start sites of 14,475 consensus coding sequences genes throughout the human genome [12]. To complement this approach, we also performed parallel genome-wide mRNA expression profiling using the Human Ht-12v3 expression beadchip, which targets more than 25,203 transcripts. These two platforms share approximately 66% genes; thus, the majority of the human genes were represented in our genome-wide analysis.

We first compared the DNA methylation patterns in uterine leiomyoma versus adjacent normal myometrium following these thresholds: fold change >2, P<0.001, and FDR (false discovery rate)<0.01. A total of 585 transcriptional regulatory regions were hypermethylated, and 446 were hypomethylated in uterine leiomyoma compared with adjacent normal myometrial tissue (Figure 1A). We then analyzed mRNA expression patterns using the following criteria: fold change >1.5, P<0.001 and FDR<0.01. We found that 307 mRNA species were downregulated, and 218 were upregulated in uterine leiomyoma compared with myometrial tissue (Figure 1A). We then analyzed the relationship between the DNA methylation of a transcriptional regulatory region and mRNA expression of the gene in that region. A total of 55 genes showed both differential DNA methylation and changes in mRNA expression in uterine leiomyoma and adjacent normal myometrial tissue (Figure 1A). Compared with the myometrium, uterine leiomyoma contained 54/55 genes (62%) that were hypermethylated and transcriptionally downregulated and 10/55 genes (18%) that were hypomethylated and transcriptionally upregulated (Figure 1B). Thus, 44/55 genes (80%) showed an inverse correlation between promoter region methylation and mRNA expression (Table 2 and 3). We also observed that 15% of the overlapping genes were hypermethylated and transcriptionally upregulated, and a much smaller number (5%) were hypomethylated and downregulated.

**Patterns of differential DNA methylation and mRNA expression in uterine leiomyoma and matched adjacent myometrial tissues**

We further analyzed the group of 55 genes that overlapped with respect to differential DNA methylation and mRNA expression. The majority of the 18 uterine leiomyoma samples exhibited a homogeneous pattern of DNA hypermethylation, whereas the normal myometrial samples were largely hypomethylated (Figure 2A). Intriguingly, while differential mRNA expression in the uterine leiomyoma and adjacent normal myometrial samples exhibited a more heterogeneous pattern (Figure 2B), the pattern was a mirror image of the differential DNA methylation pattern (Figure 2A). We also performed a functional analysis of the 55 overlapping genes using Ingenuity Pathways Analysis (IPA) and the Bioconductor GeneAnswers package, and found that based on their p-values level, the top two most significantly enriched gene functions are cancer processes (P<10<−12>) or reproductive system diseases (P<10<−8>) [13]. The genes involved in cancer were DLEC1, KRT19, KLF11, SERPINF1, TEK, APOLD1, LYZ1, CCL2, IL17B, and TNFS10, and genes involved in reproductive system diseases were CRIM1, PCP4, CHRD12, HOXA5, PLP1, COL9A2, SOX18, BMP, CALCRL, SFRP1 (Figure 2C).

**Validation of differential DNA methylation using bisulfite genomic sequencing**

We hypothesized that the 55 overlapping genes with differential DNA methylation and mRNA expression in uterine leiomyoma compared with normal myometrium were likely to be true targets of epigenetic regulation in uterine leiomyoma. Initially, we examined the regulatory CpG islands in the promoter regions of selected genes from the 55 candidates, and characterized the positions of 5’ CpG islands and transcriptional start sites using available genome databases. From this set, we then selected three of the hypermethylated genes, Kruppel-like transcription factor 11 (KLF11), deleted in lung and esophageal cancer 1 (DLEC1), keratin 19 (KRT19) for further analysis based on their known tumour suppressor functions.

First, we studied the KLF11 promoter via sequencing of bisulfite-treated genomic DNA from uterine leiomyoma and myometrial tissues from 8 subjects. Four of these were African American that were included in our original genome-wide DNA methylation study (#1–4), and we incorporated four new matched samples from Caucasian subjects (#5–8). We analyzed the DNA methylation status of a cluster of 16 CpG dinucleotides across a 249-bp region of a CpG island, located approximately −900 bp to −500 bp upstream of the KLF11 promoter region (Figure 3A). Four to six clones were sequenced from each subject. The detailed CpG methylation level of primary leiomyoma (n=8) and matched myometrial (n=8) tissues verified the hypermethylated state of the KLF11 promoter in uterine leiomyoma compared with adjacent normal myometrium. Six of the eight uterine leiomyoma samples showed increased DNA methylation of the KLF11 promoter. In
uterine leiomyoma, the majority of the 16 CpG dinucleotides in the KLF11 promoter were consistently methylated. There was a significant statistical difference (P < 0.004, Student's t-test) in DNA methylation levels between the uterine leiomyoma and matched myometrial tissues (Figure 3B).

Then, we analyzed the promoter region of another tumor suppressor gene, DLEC1, in uterine leiomyoma and myometrial samples from 7 subjects. Three subjects were African Americans included in our original genome-wide DNA methylation study (#1–3), and we incorporated four new matched samples from Caucasian subjects (#4–7). We sequenced a cluster of 18 CpG dinucleotides across a 252-bp region of a CpG island located within a −210 to +150 bp region of the DLEC1 promoter (Figure 3C). Uterine leiomyoma tissues demonstrated a dense methylation pattern at the DLEC1 promoter region in 5 of the 7 subjects. The majority of the 18 CpG dinucleotides in the DLEC1 promoter were consistently methylated in uterine leiomyoma, but not in normal myometrial tissues. Overall, there was a significant statistical difference (P < 0.003, Student's t-test) in methylation levels between uterine leiomyoma and matched normal myometrial tissues (Figure 3D).

Finally, we studied the KRT19 promoter via sequencing of bisulfite-treated genomic DNA from uterine leiomyoma and myometrial tissues from 7 subjects. Four subjects were African Americans included in our original genome-wide DNA methylation study (#1–4), and we incorporated three new matched samples from Caucasian subjects (#5–7). We analyzed the DNA methylation status of a cluster of 30 CpG dinucleotides across a 300-bp region of a CpG island, located approximately −150 bp to −150 bp around the KRT19 promoter (Figure 3F). Four to six clones were sequenced from each subject. The detailed CpG methylation level of primary leiomyoma (n = 7) and matched myometrial (n = 7) tissues verified the hypermethylated state of the KRT19 promoter in uterine leiomyoma compared with adjacent normal myometrium. All seven analyzed samples showed increased DNA methylation of the KRT19 promoter. In uterine leiomyoma, the majority of the 30 CpG dinucleotides in the KRT19 promoter were consistently methylated. There was a significant statistical difference (P < 0.001, Student's t-test) in DNA methylation levels between the uterine leiomyoma and matched myometrial tissues (Figure 3F). The Illumina platform covers 50 bp regions, whereas bisulfite sequencing evaluates a 250–300 bp region, which overlaps with the 50 bp sequence of interest. These longer fragments enhance fidelity.

Impact of DNA methylation on gene expression in human uterine leiomyoma and matched adjacent myometrial tissues

To validate that DNA methylation leads to gene silencing of the tumor suppressor genes KLF11, DLEC1, and KRT19, we assessed mRNA levels in vivo using real-time RT-PCR in uterine leiomyoma and matched myometrial tissues. We performed real-time RT-PCR on all 18 samples originally used in the microarray analysis plus we incorporated 7–10 new samples from Caucasian subjects. KLF11 mRNA levels in uterine leiomyoma (18 samples from original microarrays and 7 new Caucasian samples) were considerably lower (40%) than those in matched adjacent normal myometrial tissues (n = 25; P < 0.0001, Figure 4A). DLEC1 mRNA levels in uterine leiomyoma tissues (18 samples from original microarrays and 7 new Caucasian samples) were also significantly lower (60%) than those in matched myometrial tissues (n = 28; P < 0.0001, Figure 4B). KRT19 mRNA levels in uterine leiomyoma (18 samples from original microarray and 7 new Caucasian samples) were considerably lower (66%) than those in matched adjacent normal myometrial tissues (n = 25; P < 0.0001, Figure 4C). We have not observed any differences in mRNA levels between samples from African- and Caucasian-American subjects.

| Table 1. Descriptive characteristics of subjects. |
|-----------------------------------------------|
| Subject No | Age  | Weight (gm) | Size (cm) | No of tumors in uterus | Cycle phase (endometrium) |
|------------|------|-------------|-----------|------------------------|---------------------------|
| 1          | 49   | 1500        | 10        | 10                     | Proliferative             |
| 2          | 43   | 1000        | 9         | 10                     | Proliferative             |
| 3          | 50   | 1140        | 6         | 10                     | Proliferative             |
| 4          | 45   | 950         | 6         | 10                     | Proliferative             |
| 5          | 40   | 800         | 5         | 10                     | Proliferative             |
| 6          | 35   | 1300        | 17        | 6                      | Proliferative             |
| 7          | 40   | 1300        | 12        | 5                      | Proliferative             |
| 8          | 45   | 750         | 10        | 5                      | Proliferative             |
| 9          | 48   | 450         | 8         | 5                      | Proliferative             |
| 10         | 50   | 440         | 6         | 5                      | Proliferative             |
| 11         | 30   | 700         | 16        | 3                      | Proliferative             |
| 12         | 38   | 2400        | 16        | 3                      | Proliferative             |
| 13         | 48   | 2500        | 12        | 3                      | Proliferative             |
| 14         | 42   | 440         | 7         | 1                      | Proliferative             |
| 15         | 50   | 600         | 7         | 10                     | Secretory                 |
| 16         | 50   | 840         | 6         | 10                     | Secretory                 |
| 17         | 47   | 1050        | 11        | 1                      | Secretory                 |
| 18         | 42   | 1050        | 8         | 12                     | Disordered                |

1Size: largest diameter.  
doi:10.1371/journal.pone.0033284.t001
The effects of chemical demethylation of CpG dinucleotides on mRNA levels

To determine whether the decrease in mRNA expression of these three tumor suppressor genes is regulated by DNA methylation, primary cultured leiomyoma smooth muscle cells isolated from 7 new subjects not previously used in microarrays (4 subjects were African-American and 3 Caucasian-American) were treated with the DNMT inhibitor, 5-aza-dC at different concentrations (0.5, 1, 3, 5, 10, 15, 20 μM) and time points (1, 3, 5 days). Real-time RT-PCR was performed to measure KLF11, DLEC1, and KRT19 mRNA levels. We observed that 5-aza-dC treatments at various doses had a similar effect on restoring mRNA levels. We chose the 3 μM dose to perform the subsequent experiments because it was potentially less toxic to the cells while being maximally effective. After analyzing mRNA expression after 1, 3 and 5 days, we determined that the effect is most effective at restoring mRNA expression levels after 5 days of treatment. As shown in Figure 5, 5-aza-dC treatment of primary cultured leiomyoma smooth muscle cells led to an increase in KLF11 mRNA by 1.4-fold, DLEC1 mRNA by 2-fold, and KRT19 mRNA by 2.4-fold suggesting that these three genes are epigenetically regulated in leiomyomas.

Protein expression in human uterine leiomyoma and matched adjacent myometrial tissues

To understand the in vivo relevance of how DNA methylation affects gene function, we analyzed protein levels of KLF11, DLEC1 and KRT19 in human leiomyoma and matched normal myometrial tissues using western blot. KLF11 protein levels in all 6 subjects were significantly lower (30%) in leiomyoma compared with myometrial tissues (Figure 6A and D). Overall, DLEC1 protein levels were also significantly lower (30%) in leiomyoma than in myometrial tissues, and only 2 out of 9 subjects had no difference in DLEC1 expression in leiomyoma compared with myometrial tissues (Figure 6B and E). KRT19 protein levels in 8 subjects were lower (25%) in leiomyoma than myometrial tissues, and only 1 subject had higher KRT19 protein levels in leiomyoma compared with myometrial tissues (Figure 6C and F). All protein studies were performed with 6–9 new pairs of matched samples not previously used in the microarray experiments; 5 subjects were African American and 4 subjects were Caucasian. Overall, western blots showed that KLF11 (n=6, P<0.0001), DLEC1 (n=9, P<0.005) and KRT19 (n=8, P<0.03) (Student's t-test). Protein levels in leiomyoma tissues were significantly lower compared with matched normal myometrial tissues.

Figure 1. Integration of DNA methylation and mRNA expression data in uterine leiomyoma and matched myometrial tissues. (A) The Venn diagram integrates the number of differentially DNA methylated genes with the number of differentially expressed genes in leiomyomas compared to myometrial tissues. Approximately 55 genes showed changes in methylation status and expression. (B) A total of 34 (62%) of the genes were hypermethylated and downregulated, 10 (18%) were hypomethylated and upregulated, 8 (15%) were hypermethylated and upregulated and 3 (5%) were hypomethylated and downregulated.

doi:10.1371/journal.pone.0033284.g001
Discussion

Recent evidence suggests that DNA is differentially methylated in uterine leiomyoma versus adjacent normal myometrial tissue; however, these findings are predominantly reported in small studies and analysis of individual candidate genes such as ESR1, which has been shown to be hypomethylated in leiomyomas [9–10]. We particularly paid attention to the ESR1 gene, but we did not observe any differential DNA methylation patterns between leiomyoma and myometrium. Hypomethylation of ESR1 in leiomyoma was reported using a group of Japanese subjects; thus the difference between our findings and theirs could be attributed to racial differences. Similar racial differences have also been reported for the aromatase mRNA levels and promoter usage in uterine leiomyomas [14].

More recently published reports have attempted to demonstrate differential DNA methylation in leiomyomas; one study examined differences across the X chromosome in a single subject supporting the concept of epigenetic regulation in uterine leiomyoma. However, the other study was insufficient to identify differences in DNA methylation, which could be due to the small number of samples investigated [15–16]. Here, we report the first genome-wide DNA methylation study in uterine leiomyoma.

Table 2. Summary of hypermethylated, transcriptionally downregulated genes in uterine leiomyoma compared with adjacent normal myometrium (N = 18 matched samples).

| Gene symbol | Gene name | DNA Methylation | P-value | mRNA level | P-value |
|-------------|-----------|-----------------|---------|------------|---------|
| KRT19       | Keratin 19| 12.81           | 4.92E-10| –2.5166    | 5.68E-05|
| NUAK1       | NUAK family, SNF1-like kinase, 1 | 7.327 | 9.98E-07 | –1.5532 | 6.70E-06 |
| KLF11       | Kruppel-like factor 11 | 7.1816 | 1.40E-07 | –1.6765 | 1.32E-05 |
| DLEC1       | deleted in lung and esophageal cancer 1 | 6.3828 | 1.01E-07 | –1.51 | 1.44E-03 |
| CORIN       | Corin, serine peptidase | 5.9463 | 2.85E-09 | –1.5615 | 2.17E-05 |
| EEFMP1      | EGF-containing fibulin-like extracellular matrix protein 1 isoform b | 5.6828 | 4.02E-09 | –3.32 | 9.22E-06 |
| MBP         | Myelin basic protein | 5.5791 | 1.83E-06 | –1.7654 | 1.50E-04 |
| TMEM173     | Transmembrane protein 173 | 5.1523 | 7.89E-07 | –1.6351 | 2.56E-04 |
| TNFSF10     | Tumor necrosis factor (ligand) | 4.9483 | 1.51E-08 | –1.9423 | 1.16E-07 |
| BST2        | Bone marrow stromal cell antigen 2 | 4.5596 | 9.82E-11 | –2.1626 | 1.44E-06 |
| C1orf115    | Chromosome 1 open reading frame 115 | 3.9093 | 9.45E-07 | –1.7191 | 4.16E-08 |
| HOXA5       | Homeobox A5 | 3.7572 | 8.49E-08 | –1.7155 | 7.35E-06 |
| TEK         | TEK tyrosine kinase, endothelial | 3.7477 | 9.69E-09 | –1.8461 | 1.37E-06 |
| RB1P        | Retinol binding protein 1, cellular | 3.686 | 1.10E-08 | –2.3092 | 1.14E-04 |
| RASIP1      | Ras interacting protein 1 | 3.5885 | 1.06E-11 | –1.7365 | 9.28E-05 |
| GRAMD3      | GRAM domain containing 3 | 2.8236 | 1.19E-05 | –1.5505 | 1.03E-05 |
| CCDC109B    | Coiled-coil domain containing 109B | 2.645 | 1.60E-05 | –1.5931 | 4.92E-05 |
| APOLD1      | Apolipoprotein D domain containing 1 | 2.6009 | 1.45E-12 | –1.9577 | 6.72E-05 |
| CALCRL      | Calcitonin receptor-like | 2.5571 | 2.18E-06 | –1.856 | 3.63E-07 |
| SERPINF1    | Serpin peptidase inhibitor, clade F | 2.4166 | 3.88E-05 | –1.8676 | 9.71E-04 |
| TM4SF1      | Transmembrane 4 L6 family | 2.2707 | 7.66E-08 | –1.9102 | 3.64E-07 |
| CD34        | CD34 molecule | 2.2474 | 1.41E-09 | –1.585 | 1.51E-05 |
| CFB         | Complement factor B | 2.2336 | 3.08E-04 | –1.9627 | 4.53E-06 |
| SRGN        | Serglycin | 2.2307 | 2.10E-05 | –1.8865 | 1.33E-06 |
| LYVE1       | Lymphatic vessel endothelial hyaluronan receptor 1 | 2.2275 | 9.19E-07 | –2.4155 | 1.90E-05 |
| LCN6        | Lipocalin 6 | 2.1991 | 2.38E-10 | –1.5541 | 2.34E-06 |
| PCOLCE      | Procollagen C-endopeptidase enhancer | 2.1615 | 8.38E-05 | –2.2239 | 1.87E-05 |
| DARC        | Duffy blood group, chemokine receptor | 2.1597 | 1.44E-09 | –3.5596 | 4.90E-06 |
| CLDN5       | Claudin 5 | 2.1438 | 1.44E-09 | –1.6468 | 7.82E-05 |
| S1PR1       | Sphingosine-1-phosphate receptor 1 | 2.1421 | 1.44E-09 | –1.592 | 2.52E-06 |
| CCL21       | Chemokine (C-C motif) ligand 21 | 2.1272 | 2.65E-07 | –1.9191 | 5.97E-05 |
| HATATP2     | HIV-1 Tat interactive protein 2, 30 kDa | 2.1085 | 1.19E-04 | –1.6651 | 1.88E-06 |
| SOX18       | SRY (sex determining region Y)-box 18 | 2.0969 | 9.81E-08 | –1.699 | 5.79E-05 |
| CREG1       | Cellular repressor of E1A-stimulated genes 1 | 2.0799 | 4.55E-05 | –1.6091 | 3.35E-05 |
| PECA1       | Platelet/endothelial cell adhesion molecule | 2.0659 | 3.79E-08 | –1.6298 | 9.27E-06 |
| CRIM1       | Cysteine rich transmembrane BMP regulator 1 | 2.0092 | 1.84E-08 | –1.6596 | 1.51E-05 |

1Fold change was calculated as mean methylation beadchip value for leiomyoma relative to normal myometrium.
2Fold change was calculated as mean mRNA expression microarray value for leiomyoma relative to normal myometrium.
doi:10.1371/journal.pone.0033284.t002
Table 3. Summary of hypomethylated, transcriptionally upregulated genes in uterine leiomyoma compared with adjacent normal myometrium (N = 18 matched samples).

| Gene symbol | Gene name                          | DNA Methylation | P-value   | mRNA level | P-value   |
|-------------|------------------------------------|-----------------|-----------|------------|-----------|
| POPDC2      | Popeye domain containing 2         | 0.0818          | 3.73E-07  | 1.7758     | 1.14E-04  |
| PCP4        | Purkinje cell protein 4            | 4.2627          | 3.38E-08  | 3.2515     | 2.91E-04  |
| EL17B       | Interleukin 17B                    | 4.0467          | 1.66E-08  | 3.0582     | 3.46E-06  |
| CHRDLD2     | Chordin-like 2                     | 2.8997          | 3.19E-06  | 2.8911     | 1.43E-04  |
| RPE65       | Retinal pigment epithelium-specific| 2.8382          | 3.57E-06  | 1.623      | 6.00E-04  |
| PHDB2       | Pleckstrin homology-like domain,   | 2.6662          | 1.11E-10  | 1.5018     | 5.80E-05  |
| MMP11       | Matrix metalloproteinase 11        | 2.585           | 9.82E-07  | 4.921      | 1.85E-04  |
| MFAP2       | Microfibrillar-associated protein 2| 2.2917          | 4.96E-06  | 2.1194     | 2.81E-06  |
| JPH4        | Junctophilin 4                     | 2.2868          | 5.50E-08  | 1.5724     | 3.39E-06  |
| PLP1        | Proteolipid protein 1             | 2.1274          | 4.06E-04  | 3.3833     | 6.71E-05  |

1. Fold change was calculated as mean methylation beadchip value for leiomyoma relative to normal myometrium.
2. Fold change was calculated as mean mRNA expression microarray value for leiomyoma relative to normal myometrium.

doi:10.1371/journal.pone.0033284.t003

Wide analysis of differential DNA-methylation and mRNA expression in uterine leiomyoma and adjacent normal myometrial tissues from 18 matched pairs, all from African American subjects to limit biological heterogeneity, and avoid epigenetic variations among ethnic groups. The following real-time RT-PCR validation of mRNA expression and bisulfite sequencing validation of DNA methylation of these genes were performed on both a subset of the original African American samples and additional new samples from Caucasian subjects. Additionally, in vitro cultured experiments utilized primary cells from both ethnic groups. We have not observed any apparent differences with respect to the 3 studied genes between samples from African- and Caucasian-American subjects suggesting that the findings may be applicable to both ethnic groups. This conclusion, however, should be taken with some caution due to the low number of Caucasian subjects. Further studies are needed to make a more definitive conclusion.

Our study confirms the link between epigenetic DNA modifications and gene expression in uterine leiomyomas, by demonstrating the effects of promoter DNA methylation on gene silencing, particularly in three tumor suppressors known to be involved in reproductive tumorigenesis. Though our work is the first to examine genome-wide analysis of DNA methylation in uterine leiomyoma and there are no existing data against which we can compare our results, our mRNA expression profiles are consistent with previously published reports [17–26].

In this study, we noted a key epigenetic mechanism whereby increased promoter methylation leads to transcriptional suppression in uterine leiomyoma compared with matched normal myometrial tissues. The second predominant mechanism was hypomethylation associated with overexpression of genes indicating an overall inverse relationship between DNA methylation and gene expression in uterine leiomyoma. However, we also observed some genes to be hypermethylated and upregulated, and other genes to be hypomethylated and downregulated. The absence of an inverse relationship between promoter DNA methylation and mRNA expression in this minor group of genes is consistent with previously published data. For example, methylation of one particular CpG island in the NR5A1 gene is associated with transcriptional suppression, whereas methylation of another CpG island located 4 kb downstream is associated with overexpression of NR5A1 mRNA [17,27]. It is conceivable that the effects of a single methylated CpG island on gene expression may be either gene-specific or location-specific within the same gene.

We verified the effects of promoter DNA methylation on transcriptional inhibition of three tumor suppressor genes namely, KLF11, DLEC1, and KRT19. KLF11 is a transcription factor and a member of the transforming growth factor beta (TGFβ) family, which is involved in key cellular functions such as apoptosis, proliferation, and differentiation [28]. KLF11 is expressed in a number of human tissues, and it is repressed in several human cancers. It inhibits neoplastic transformation and cell growth both in vivo and in vitro [29]. We previously demonstrated the downregulation of KLF11 expression in uterine leiomyoma tissues compared with normal matched myometrial tissue [30]. Although the mechanism involved in KLF11-regulated cell proliferation is not fully understood, we demonstrated for the
Genome-Wide DNA Methylation in Uterine Leiomyoma

A. Leiomyoma  Myometrium

B. Leiomyoma  Myometrium

C.

Cancer
Reproductive Diseases
Cardiovascular Development & Function
Organisinal Development
Hematological Disease
Cell-mediated Immune Response
Cellular Movement
Hematological Development & Function
Immune Cell Trafficking
Cell Morphology
Cell-to-Cell Signaling and Interaction
Cell Death
first time that KLF11 is epigenetically regulated by DNA methylation, with hypermethylation correlating with a repressed state in uterine leiomyoma. Recently, KLF11 was also shown to be aberrantly hypermethylated in myelodysplastic syndromes. It has been suggested that KLF11 inhibits gene expression through a Sin3a-HDAC interacting domain and recruitment of the corepressor mSin3a [31]. We plan to investigate this mechanism further, and identify the DNMTs and DNA methyl binding proteins that are involved in silencing of KLF11.

DLEC1 is an epigenetically modified tumor suppressor gene [32]. DLEC1 is localized in the cytoplasm ubiquitously expressed in all human tissues, and repressed in several human cancers. Hypermethylation of the DLEC1 promoter is associated with its transcriptional repression in a wide variety of malignant tumors originating from lung, esophagus, kidney, ovary, nasopharynx, and liver [33]. The DLEC1 promoter region contains a CpG island in the first exon, and we demonstrated here that methylation of this CpG is responsible for the repression of DLEC1 expression in uterine leiomyoma. Our analysis revealed a strong association between silencing of DLEC1 expression and promoter hypermethylation in uterine leiomyoma; in addition, treatment of addition of cultured primary uterine leiomyoma smooth muscle cells with a DNMT inhibitor restored DLEC1 expression. The DLEC1 gene encodes a 166 kDa protein, whose biologic function remains unknown due to lack of homology to any known conserved proteins or domains [34]. In the future, we plan to characterize the biological function of DLEC1 in uterine leiomyoma.

KRT19 is an intermediate filament protein responsible for the structural integrity of epithelial cells, this genes encodes a 40-kDa protein [35]. In mammalian cells, keratin filaments are organized in a complex network spreading from the nucleus to the cytoplasmic membrane. KRT19 is also known as an epigenetically regulated tumor suppressor gene, which has frequently demonstrated promoter hypermethylation associated with transcriptional downregulation in several cancerous tumors such as neuroblastomas, squamous cell carcinoma of the head and neck region and renal cell carcinomas [36–38]. Also, it is one of the most common used markers for real-time RT-PCR detection of tumor cells disseminated in lymph nodes, peripheral blood and bone marrow of breast cancer patients [39–40].

Using genome-wide analyses of DNA methylation in uterine leiomyoma we hope to define a specific epigenetic profile that could inform the development of diagnostic biomarkers for uterine leiomyoma as well as identify potential therapeutic targets. Because DNA methylation is reversible, epigenetic modifying drugs could be used in the medical management of uterine leiomyoma. Importantly, aberrant DNA methylation and other epigenetic abnormalities may represent a critical initial mechanism that triggers transformation of a single myometrial cell that will eventually give rise to a monoclonal leiomyoma tumor. Understanding the mechanism underlying the pathogenesis of uterine leiomyoma will be critical for developing new preventive and therapeutic approaches to the disease.

Materials and Methods

Ethics Statement

To obtain human tissues, we followed the protocol approved by the Institutional Review Board for Human Research of Northwestern University and New York University. Written informed consent was received from all subjects.

Tissue acquisition

For in vivo studies, we obtained matched pairs of leiomyoma and adjacent myometrium from a total of 23 African American and 14 Caucasian-American subjects undergoing hysterectomy for symptomatic fibroids. To minimize heterogeneity due to race we used samples from 18 African American subjects for both genome-wide DNA methylation and gene expression microarrays. In follow-up verification studies, we included samples from 4 of the original African American group plus 4 additional Caucasian subjects for bisulfite sequencing and all 18 original African American plus 10 Caucasian subjects for mRNA quantification using real-time RT-PCR. Samples from Caucasian subjects were added to evaluate whether similar patterns of DNA methylation and mRNA expression were observed.

Key clinical characteristics of the 18 African American subjects, whose samples were used for both microarrays are described in Table 1. The clinical characteristics of Caucasian subjects fall within the range depicted in Table 1. All African- or Caucasian-American subjects were premenopausal women (mean age 44 years; range 30–50 years), and the tumor sizes ranged from 5 to 17 cm in diameter. The subjects had not received any hormonal treatment for at least 6 months prior to surgery. The cycle phase was estimated by the last menstrual period and was confirmed by endometrial histology. Each leiomyoma tissue biopsy was obtained at 1 cm from the outer capsule of the tumor. The matched myometrial sample was taken at 2 cm from the tumor in the fundal portion of the uterus. All tissue samples were snap-frozen in liquid nitrogen before DNA and RNA isolation. The leiomyoma and myometrial tissue specimens were biopsied by a pathologist under the supervision of Dr. Wei, who is a board-certified pathologist. Dr. Wei and his pathology team carefully examined these specimens both grossly and histologically and made sure that they were not contaminated by endometrial stroma or epithelium.

The initial sample size of 18 pairs for microarray platforms was derived from the power analysis based on published clinical studies, which showed that significant differences for most dysregulated gene products can be detected with sample size of 13 to 22 subjects per group with 80% power and 0.05 alpha levels [41].

Primary cell isolation

Leiomyoma smooth muscle cells were isolated from the peripheral portions approximately 1 cm from the outer capsule of the leiomyoma, and then cultured as previously described with minor modifications [42]. Cells were cultured in DMEM/F12 1:1 (GIBCO/BRL, Grand Island, NY) containing 10% fetal bovine

Figure 2. Differential patterns of DNA methylation and mRNA expression in uterine leiomyoma and matched myometrial tissues. Overall, 36 samples from 18 subjects were analyzed; 18 samples were obtained from leiomyomas and 18 from adjacent matched myometrial tissues. (A) The DNA methylation profile shows that most of the samples exhibit a very homogeneous DNA methylation pattern. (B) The gene expression profile exhibits a less homogeneous pattern, which could be due to mRNA instability. (C) Functional analysis of the 55 genes with correlation between DNA methylation status and mRNA expression showed that the top two functions represented are cancer processes (P < 10^{-12}) and reproductive system disease (P < 10^{-8}). doi:10.1371/journal.pone.0033284.g002
serum (Invitrogen, Carlsbad, CA) and grown in a humidified atmosphere with 5% CO2 at 37°C. Primary cells were used only up to the second passage to avoid changes in phenotype and gene expression.

DNA methylation and mRNA expression analysis

Genomic DNA was isolated from 20 mg frozen tissues using the DNeasy Blood & Tissue (Qiagen, Valencia, CA). One microgram of genomic DNA from each sample was bisulfite-modified using

Figure 3. DNA methylation status of KLF11, DLEC1, and KRT19 promoters in uterine leiomyoma and matched myometrial tissues. (A) DNA methylation status of 16 CpG sites in the KLF11 promoter region obtained from bisulfite sequencing of uterine leiomyoma and matched myometrial tissues. The numbers 1 to 8 on the side represent subjects from whom tissues were obtained, (#1–4) subjects were African Americans included in our original genome-wide DNA methylation study and we incorporated 4 new matched samples from Caucasian subjects (#5–8). Black circles represent methylated cytosines and white represent unmethylated cytosines. Numbers indicate the position of cytosine residues of CpGs relative to the transcription start site (+1). (B) Percent DNA methylation of the KLF11 promoter region in uterine leiomyoma and myometrial tissues, P<0.003. (C) DNA methylation status of 18 CpG sites in the DLEC1 promoter region from 3 African American (#1–3) and 4 Caucasian subjects (#4–7). (D) Percent DNA methylation of the DLEC1 promoter region in uterine leiomyoma and myometrial tissues, P<0.003. (E) DNA methylation status of 30 CpG sites in the KRT19 promoter region from 4 African American (#1–4) and 3 Caucasian subjects (#4–7). (F) Percent DNA methylation of the KRT19 promoter region in uterine leiomyoma and myometrial tissues, P<0.001.

doi:10.1371/journal.pone.0033284.g003
the EZ DNA Methylation kit (Zymo Research, Orange, CA), according to the manufacturer’s protocol along with the technical validation of the assay [12]. Bisulfite-modified DNA was hybridized to the HumanMethylation27 Beadchip (Illumina Inc., San Diego, CA).

Total RNA was isolated from 20 mg of frozen tissues using the RNeasy Fibrous Tissue kit (Qiagen) according to manufacturer protocols with minor modifications. After elution, RNA samples were quantified using a ND-1000 spectrophotometer (NanoDrop Wilmington, DE) and evaluated for degradation using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). For use in hybridization, samples were required to have a RIN $\geq 9$, an $\text{OD}_{260/280}$ of 1.9–2.0, and $\text{OD}_{260/230} \leq 1.5$, and a 28S:18S ribosomal band ratio of $\geq 1.5$. The samples were hybridized into the HumanHT-12 v3 genome-wide gene expression BeadChips according to the manufacturer’s protocol (Illumina, Inc.).

We used the Bioconductor lumi [43] package, which was developed by our collaborator and is widely used as one of the standard tools to process both Illumina DNA methylation and mRNA expression data. The data first went through a QA/QC step. For Illumina expression data, the data passing QA step was preprocessed using a variance stabilization transformation method [44] followed by quantile normalization. For methylation data, we first performed a color balance adjustment of methylated and unmethylated probe intensities between two color channels using a smooth quantile normalization method. The methylated and unmethylated probe intensities were then normalized using the SSN (Scale and Shift Normalization) method. The methylation M-value (log 2 ratio of methylated and unmethylated probes) was calculated to estimate the methylation level of the measured CpG sites [45]. The follow-up analysis was then based on the M-value. We used a shift and scaling normalization (SSN) method, which includes global background shift during normalization instead of more aggressive quantile normalization as described in reference 45. We made this decision primarily because we produced high quality and consistent data evident by the principal component analysis that we are now incorporating in the supplemental section.

After preprocessing, the differential analysis of methylation data was similar to that used for expression microarray data. Probes (for expression data) or CpG-sites (for methylation data) with all samples “Absent” (lower or around background levels) were removed from analysis.

Figure 4. mRNA levels of KLF11, DLEC1 and KRT19 in uterine leiomyoma and matched adjacent myometrial tissues. mRNA levels of (A) KLF11, (B) DLEC1, and (C) KRT19 were quantified using all 18 samples used in microarrays plus the addition of 7–10 new Caucasian samples. mRNA levels in leiomyoma and matched myometrium were quantified by real-time PCR, they were first normalized to GAPDH. Then, to allow comparisons of data obtained from samples from different subjects, mRNA levels in the myometrial tissues were normalized to 1. KLF11 ($n = 25$; $P < 0.0001$), DLEC1 ($n = 28$; $P < 0.0001$) and KRT19 ($n = 25$; $P < 0.0001$). The data are shown as the mean $\pm$ SEM. $P < 0.05$ versus myometrium tissues. doi:10.1371/journal.pone.0033284.g004

Figure 5. Effect of DNMT inhibitor 5-aza-dC on KLF11, DLEC1 and KRT19 expression in leiomyoma smooth muscle cells. (A) KLF11, (B) DLEC1 and (C) KRT19 mRNA levels were quantified by real-time RT-PCR after treatment of cultured uterine leiomyoma smooth muscle cells with vehicle or 5-aza-dC (3 $\mu$M) for 5 days. mRNA levels were normalized first to GAPDH and then, to those in vehicle-treated cells. Experiments were performed in triplicate samples, and the data are representative of samples from seven different subjects. The data are shown as the mean $\pm$ SEM. $P < 0.05$ as compared with vehicle treatment. doi:10.1371/journal.pone.0033284.g005
BIOSERVICES, Foster City, CA) and the ABI Prism 7900HT Detection System (Applied Biosystems). Cycling conditions started at 50°C for 2 min, followed by 95°C for 10 min, then 40 cycles of 95°C for 15 sec and 60°C for 1 min. The cycle threshold (Ct) was placed at a set level where the exponential increase in PCR amplification was approximately parallel between all samples. Relative fold change was calculated by comparing Ct values between the target gene and GAPDH as the reference guide. The primer sequences used were:

KL11: forward: 5'-CAGGATGCAACACGGCGGAC-3'; reverse: 5'-TGGCTGTGCTAGCCGCTTCTC-3'; DLEC1: forward: 5'-GAGCAAGTTGAGGCCAAGC-3'; reverse: 5'-ATCCAGCCGCTGGTTATAGA-3'; GAPDH: forward: 5'-GAAGGTGAAGG-CTGCTTATAGA-3'; reverse: 5'-GAGATGGTGATGGGATTTC-3'.

The ΔΔCt method was used to analyze the relative changes in gene expression.

5-aza-2'-deoxycytidine (5-aza-dC) treatment
Monolayer cultures at approximately 40% confluence were starved in serum-free medium overnight and treated with vehicle (DMSO 1:1000) or 0.5, 1, 3, 5, 10, 15, or 20 μM of the DNMT inhibitor 5-aza-dC (Sigma-Aldrich) for 5 days. The medium was changed every 24 hrs. Total RNA was isolated using TRI-reagent (Sigma-Aldrich). All of the experiments were repeated in triplicate using samples from at least 7 new different subjects not previously used in microarrays. 4 subjects were African- and 3 Caucasian-American.

Protein Analysis
Protein was extracted from 50 mg of frozen tissues using mammalian protein extraction reagent (Pierce, Rockford, IL).
Lysates were cleared by centrifugation at 14,000 rpm for 10 min. Equal amounts of protein (30 ug) were resolved on 4–12% Ready Gel Precast Gels (Bio-Rad Laboratories, Hercules, CA), and transferred onto PVDF membranes. The membranes were blotted with antihuman KLF11 antibodies (1:1000 (Cell Signalling), DLEC1 1:500 (Sigma-aldrich) and KRIT1 1:1000 (Cell Signalling). Anti-GAPDH antibody was used as a loading control. Detection was detected using a Supersignal West Femto (Pierce). Quantification of the immunoblots was done using ImageJ software and normalized to GAPDH.

Statistical analysis

Statistical significance was determined by Student’s t test and one-way ANOVA followed by Fisher’s protected least significant difference test. Significance was accepted at P<0.05.

Author Contributions

Conceived and designed the experiments: AN SML SEB. Performed the experiments: AN. Analyzed the data: AN PD SML SEB. Contributed reagents/materials/analysis tools: PY DM JW. Wrote the paper: AN SEB.

References

1. Andersen J, Barbieri RL (1995) Abnormal gene expression in uterine leiomyomas. J Soc Gynecol Investig 2: 663–672.
2. Kjerulff KH, Langenberg P, Seidman JD, Stolley PD, Guzinski GM (1996) Uterine leiomyomas. Racial differences in severity, symptoms and age at diagnosis. J Reprod Med 41(7): 483–90.
3. Baird DD, Dunson DB, Hill MC, Coumans D, Schectman JM (2003) High cumulative incidence of uterine leiomyoma in black and white women: ultrasound evidence. Am J Obstet Gynecol 181(1): 100–7.
4. Haney AF (2000) Clinical decision making regarding leiomyomata: what we know for the next millennium. Environ Health Perspect 108 Suppl 1: 113–17.
5. Flynn M, Jamison M, Datta S, Myers E (2006) Health care resource use for uterine leiomyoma. PLoS One 1: e2152.
6. Ligon AH, Morton CC (2001) Leiomyomata: heritability and cytogenetic studies. Hum Reprod Update 7: 8–14.
7. Grafi G, Zemach A, Pinno L (2007) Methyl-CpG-binding domain (MBD) proteins in plants. Biochim Biophys Acta 1769: 207–294.
8. Li S, Chiang TC, Richard-Davis G, Barrett JC, McLachlan JA (2003) DNA microarrays and gene expression analysis. Int J Exp Pathol 84: 267–279.
9. Wei T, Geiser AG, Qian HR, Su C, Helvering LM, et al. (2007) DNA methylation and imbalanced expression of DNA methyltransferases (DNMT1, 3A, and 3B) in human uterine leiomyoma. Gynecol Oncol 98: 125–130.
10. Yamagata Y, Asada H, Tamura I, Lee L, Maekawa R, et al. (2009) DNA methyltransferase expression in the human endometrium: down-regulation by progesterone and estrogen. Hum Reprod 24: 1126–1132.
11. Asada H, Yamagata Y, Takeuti T, Matsuo A, Tamura H, et al. (2008) Potential link between estrogen receptor-alpha gene hypomethylation and uterine fibroid formation. Mol Hum Reprod 14: 539–545.
12. Bibikova M, Fan JB (2009) GoldenGate assay for DNA methylation profiling. Methods Mol Biol 507: 149–163.
13. Feng G, Du P, Krett NL, Tessl M, Rosen S, et al. (2010) A collection of bioconductor methods to visualize gene-list annotations. BMC Res Notes 3: 10.
14. Ishikawa H, Reierstad S, et al. (2009) High aromatase expression in uterine leiomyoma tissues of African-American women. J Clin Endocrinol Metab 94(5): 1734–1740.
15. Maekawa R, Yagi S, Ohsue J, Yamagata Y, Asada H, et al. (2011) Disease-dependent Differently Methylated Regions (D-DMRs) of DNA are Enriched on the X Chromosome in Uterine Leiomyoma. J Reprod Dev. 57: 290–298.
16. Cai LY, Izumi S, et al. (2011) Does aberrant DNA methylation occur in human uterine leiomyomas? An attempt of genome-wide screening by MS-RDA. Tokai J Exp Clin Med 36(3): 84–88.
17. Xue Q, Liu Z, Yin P, Miled MP, Cheng YH, et al. (2007) Transcriptional activation of steroidogenic factor-1 by hypomethylation of the 5′ CpG island in endometriosis. J Clin Endocrinol Metab 92: 3261–3267.
18. Arslan AA, Gold LI, Mittal K, Suen TC, Belitskaya-Levy I, et al. (2005) Gene expression studies provide clues to the pathogenesis of uterine leiomyoma: new evidence and a systematic review. Hum Reprod Update 7: 8–14.
19. Ishikawa H, Reierstad S, et al. (2009) High aromatase expression in uterine leiomyoma tissues of African-American women. J Clin Endocrinol Metab 94(5): 1734–1740.
20. Tsibris JC, Segars J, Coppola D, Mane S, Wilbanks GD, et al. (2002) Insights into the development of cellular markers for uterine leiomyoma. Fertil Steril 78: 114–121.
21. Catterino WH, Prupas C, Tsibris JC, Leppert PC, Payson M, et al. (2003) Strategy for elucidating differentially expressed genes in leiomyoma identified by microarray technology. Fertil Steril 80: 202–209.
22. Xu H, Liu Z, Yin P, Miled MP, Cheng YH, et al. (2007) Transcriptional activation of steroidogenic factor-1 by hypomethylation of the 5′ CpG island in endometriosis. J Clin Endocrinol Metab 92: 3261–3267.
23. Arslan AA, Gold LI, Mittal K, Suen TC, Belitskaya-Levy I, et al. (2005) Gene expression studies provide clues to the pathogenesis of uterine leiomyoma: new evidence and a systematic review. Hum Reprod Update 7: 8–14.
24. Hofmann PF, Miliken DB, Gregg LC, Davis RR, Gregg JP (2004) Molecular characterization of uterine fibroids and its implication for underlying mechanisms of pathogenesis. Semin Reprod Med 22(3): 207–215.
25. Quade BJ, Wang TY, Sorberker B, Dal Cin P, Mutter GL, et al. (2004) Molecular pathogenesis of uterine smooth muscle tumors from transcriptional profiling. Genes Chromosomes Cancer 40: 97–106.
26. Skubitz KM, Skubitz AP (2000) Differential gene expression in uterine leiomyomas. J Lab Clin Med 141: 297–308.
27. Xue Q, Zhou YF, et al. (2011) Hypermethylation of the CpG island spanning from exon II to intron III is associated with steroidogenic factor 1 expression in an immortal cell line derived from uterine leiomyoma. Int J Mol Med 27(1): 13–18.
28. Feng G, Du P, Krett NL, Tessel M, Rosen S, et al. (2010) A collection of bioconductor methods to visualize gene-list annotations. BMC Res Notes 3: 10.