Dynamic Changes in Gene Expression that Occur during the Period of Spontaneous Functional Regression in the Rhesus Macaque Corpus Luteum

Randy L. Bogan, Melinda J. Murphy, and Jon D. Hennebold
Division of Reproductive Sciences (R.L.B., M.J.M., J.D.H.), Oregon National Primate Research Center, Beaverton, Oregon 97006; and Department of Obstetrics & Gynecology (J.D.H.), Oregon Health & Science University, Beaverton, Oregon 97239

Luteolysis of the corpus luteum (CL) during nonfertile cycles involves a cessation of progesterone (P4) synthesis (functional regression) and subsequent structural remodeling. The molecular processes responsible for initiation of luteal regression in the primate CL are poorly defined. Therefore, a genomic approach was used to systematically identify differentially expressed genes in the rhesus macaque CL during spontaneous luteolysis. CL were collected before (d 10–11 after LH surge, mid-late (ML) stage) or during (d 14–16, late stage) functional regression. Based on P4 levels, late-stage CL were subdivided into functional-late (serum P4 > 1.5 ng/ml) and functionally regressed late (FRL) (serum P4 < 0.5 ng/ml) groups (n = 4 CL per group). Total RNA was isolated, labeled, and hybridized to Affymetrix genome microarrays that contain elements representing the entire rhesus macaque transcriptome. With the ML stage serving as the baseline, there were 681 differentially expressed transcripts (>2-fold change; P < 0.05) that could be categorized into three primary patterns of expression: 1) increasing from ML through FRL; 2) decreasing from ML through FRL; and 3) increasing ML to functional late, followed by a decrease in FRL. Ontology analysis revealed potential mechanisms and pathways associated with functional and/or structural regression of the macaque CL. Quantitative real-time PCR was used to validate microarray expression patterns of 13 genes with the results being consistent between the two methodologies. Protein levels were found to parallel mRNA profiles in four of five differentially expressed genes analyzed by Western blot. Thus, this database will facilitate the identification of mechanisms involved in primate luteal regression. (Endocrinology 150: 1521–1529, 2009)
species (9), possibly due to an enhanced level of basal protein kinase A activity (9, 10). Moreover, although it has been demonstrated that prostaglandin F2α (PGF2α) released from the uterus initiates functional regression in rodent and domestic animal species (8, 11), the functional lifespan of the primate CL is not extended by hysterectomy (12, 13). Numerous studies have demonstrated that intraluteal infusion of PGF2α causes luteolysis in monkeys and women (14–19), however, administration of a prostaglandin synthesis inhibitor to monkeys beginning at the midluteal phase inhibited P4 secretion and shortened CL lifespan rather than delaying luteolysis, as might have been predicted (20). Thus, the source and physiological role for PGF2α in spontaneous luteolysis of primates, if any, have not been determined (8, 11). Collectively, these studies indicate that gonadotropin and prostaglandin-induced regulation of CL function and lifespan is very different between primate and nonprimate species, and studies to understand the control of luteolysis in primates are needed.

In addition to reduced responsiveness to LH, the resultant loss in P4 synthesis may further promote luteolytic events because accumulating data indicate that P4 itself mediates many processes necessary for maintaining the structure and function of the CL (2). Treatment of monkeys with the 3β-hydroxysteroid dehydrogenase (HSD3B) inhibitor trilostane during the midluteal phase inhibited P4 secretion and shortened CL lifespan, despite restoration of normal adrenal function after cessation of treatment (21). Another steroid ablation/progesterin replacement study demonstrated that P4 mediates many of the effects of CG to rescue the CL from luteolysis during early pregnancy (22). Given that the first event in luteolysis is a cessation of P4 production and that the subsequent loss in P4-mediated signaling is likely a prerequisite for structural regression, we hypothesized that there are differences in gene expression just before and immediately after the onset of spontaneous functional regression that may represent proteins/pathways responsible for the loss of P4 producing potential in the primate CL. Previously, we identified differentially expressed genes occurring in CL collected during the early, mid, mid-late, late, and very late stages of the luteal phase of normal menstrual cycles (23). The late stage corresponds to the period when functional regression occurs. In this previous microarray study, some of the CL within this stage had not yet undergone functional regression, as indicated by high-serum levels of P4 at CL collection (>5 ng/ml in some cases). Therefore, we hypothesized that if late-stage CL were analyzed based on their functional state (as indicated by serum levels of P4), gene changes more closely associated with functional regression, and separate from downstream changes during structural regression, could be delineated. To perform the current study, additional CL were collected between d 10 and 12 (mid-late stage, n = 8 CL) of the luteal phase. The late-stage CL were further divided into two groups (n = 4 CL per group) based on serum concentrations of P4. CL from animals with serum levels of P4 more than 1.5 ng/ml were considered nonregressed, and CL from animals with serum levels of P4 less than 0.5 ng/ml were considered to have already undergone functional regression. Serum levels of LH were determined by the Oregon National Primate Research Center Endocrine Services Laboratory using a specific RIA as previously described (25, 26).

### Microarray analysis

Total RNA was extracted using TRIzol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer’s instructions and further purified using RNeasy spin columns (Qiagen, Inc., Valencia, CA). Final RNA concentrations and purity were determined by spectrophotometry, and the integrity of RNA samples was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA). Microarray assays using GeneChip Rhesus Macaque Genome Arrays (Affymetrix, Inc., Santa Clara, CA; >47,000 transcripts represented) were performed by the Affymetrix Microarray Core of the Oregon Health & Science University Gene Microarray Shared Resource. A detailed description of microarray assay procedures and subsequent generation of processed image files has been reported previously (23). See supplemental data, which is published as supplemental data on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org, for all information necessary for Minimum Information About Microarray Experiments compliance. The data discussed in this publication have been deposited in the National Center for Biotechnology Information’s Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and are accessible through Gene Expression Omnibus Series accession no. GSE12807.

The processed image files (.cel) were normalized across arrays using the robust multichip average (RMA) algorithm (27) and log transformed (base 2), thus allowing direct comparison of probe set values between all samples used in the experiment. After normalization, GeneSifter (VizX Labs, Seattle, WA) microarray expression analysis software was used to identify differentially expressed transcripts. With the mid-late CL serving as the baseline reference, transcripts that exhibited a greater than 2-fold change (ANOVA, P < 0.05; Benjamini and Hochberg correction for false discovery rate) were considered differentially expressed. In pairwise comparisons, transcripts that were more than 2-fold different between groups (t test, P < 0.05) were considered differentially expressed. The GeneSifter microarray analysis software only determines whether there is a statistically significant change relative to the baseline but does not specify at which stage(s) this occurs. Therefore, to facilitate in-depth comparison of the microarray results, Affymetrix Expression Console version 1.0 software was used to generate raw data files (.celh) that were normalized with the robust multichip average algorithm and log (base 2) transformed. Selected transcripts were then analyzed by one-way
ANOVA and pairwise differences determined with the Student-Newman-Keuls test (P < 0.05).

The list of differentially expressed genes generated by GeneSifter was divided into groups based on similarities in expression patterns. Ontology reports (molecular function) were generated from the various groups and assigned a z score by GeneSifter, with a score greater than 2.0 and a minimum of five genes being considered significant (28). Ontologies with more than 40 genes were considered too general. Significant ontologies were divided into primary and sub-ontologies. An ontology was defined as a sub-ontology if the majority of its genes were included in another significant ontology that had a greater total number of differentially expressed genes.

**TaqMan quantitative PCR (Q-PCR)**

Generation of cDNA and Q-PCR procedures were performed as described previously (23). Primer and TaqMan MGB probe sequences for the LH receptor (LHCR), HSD3B2 Δ5/Δ4 isomerase 2 (HSD3B2), cytochrome P450, family 19, subfamily A, polypeptide 1 (CYP19A1), microsomal prostaglandin E2 (PGE2) synthase-1 (PTGES), PGE2α receptor (PTGFR), and hydroxysteroid dehydrogenase 15-NAD (HPGD) have been reported (23). Forward and reverse primer, as well as MGB-probe sequences for the steroidogenic acute regulatory protein (STAR), low-density lipoprotein receptor (LDLR), scavenger receptor type B class 1 (SCARB1), ATP-binding cassette (ABC) family members A1 and G1, and apolipoprotein subfamily L (APOL) members 2 and 4 are provided in supplemental Table 1.

Relative concentrations of the target genes were normalized to 18s rRNA levels, and the ratios were log transformed before statistical analysis. Data were analyzed using one-way ANOVA, followed by pairwise comparisons with the Student-Newman-Keuls test, and differences were considered statistically significant at P < 0.05.

**Western blot analysis**

Whole cell homogenates were prepared from frozen (−80 C) CL as described previously (23). Antibodies against STAR (Affinity BioReagents, Inc., Golden, CO; catalog no. PA1-560), HSD3B2 and β-tubulin (TUBB) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; catalog nos. sc-30821 and sc-9104, respectively), CYP19A1 (Novus Biologicals, Littleton, CO; catalog no. NB100-1596), and PTGES and HPGD (Cayman Chemical Co.; Ann Arbor, MI; catalog nos. 160140 and 160615, respectively) were purchased commercially.

Either 25 μg (HSD3B2), 30 μg (STAR, PTGES, and HPGD), or 40 μg (CYP19A1) of total protein was resolved on either 10% (HSD3B2) or 4–15% gradient Tris-HCl gels. Western blot procedures were similar to those described previously (23). Antibodies were used at the following concentrations: anti-STAR, 0.65 μg/ml; anti-HSD3B2, 0.3 μg/ml; anti-CYP19A1, 0.8 μg/ml; anti-PTGES, 0.4 μg/ml; and anti-HPGD, 2 μg/ml. Specificity was determined by estimating molecular mass via comparison of bands with molecular mass markers, as well as preventing band detection by preabsorbing the primary antibody with immunizing peptide if available (HSD3B2, PTGES, and HPGD) (23, 29), or exclusion of primary antibody if no peptide was available (CYP19A1). The specificity of the anti-STAR antibody has been determined previously (10). After detection of target protein, membranes were reprobed for TUBB (0.2 μg/ml) to serve as a loading control.

Films were scanned, and densitometry analysis was performed using Quantity One version 4.3.1 software (Bio-Rad Laboratories, Inc., Hercules, CA). The background-adjusted volume of each band was normalized to TUBB, and data were log transformed if necessary for statistical normalization. The ratio of target protein to TUBB for each stage was analyzed by one-way ANOVA, followed by pairwise comparison using the Student-Newman-Keuls test with differences considered significant at P < 0.05.

**Results**

**Serum concentrations of P4 and LH**

There was no significant difference between mid-late and functional late CL in serum concentrations of P4 (4.4 ± 0.6 and 5.4 ± 1.2 ng/ml, respectively). However, functionally regressed late CL had significantly (P < 0.05) lower serum concentrations of P4 (0.4 ± 0.1 ng/ml) compared with all other groups. There were no significant differences between groups in serum levels of LH (1.0 ± 0.5 ng/ml mid-late; 2.1 ± 0.7 ng/ml functional late; and 0.7 ± 0.2 ng/ml functionally regressed late), indicating that the loss of P4 secretion in functionally regressed late CL is not due to differences in LH levels at CL collection.

**Identification of differentially expressed genes throughout functional regression of the macaque CL**

From the mid-late through functionally regressed late stages, 681 transcripts met the criteria for differential expression (>2-fold change; ANOVA, P < 0.05). After performing pairwise comparisons, 305 transcripts (>2-fold change; t test, P < 0.05) were differentially expressed between mid-late and functional late CL, of which 233 were increased, and 72 were decreased in expression. There were 314 differentially expressed transcripts
in the pairwise comparison between functional and functionally regressed late-stage CL, with 102 increasing and 212 decreasing in expression.

Cluster and scatter-plot analysis of the three experimental groups indicated that the mid-late and functional late CL were the most similar in terms of gene expression profiles, with the functionally regressed CL being the most unrelated (supplemental Fig. 1). There were three primary ontologies (oxidoreductase activity, cytoskeletal protein binding, protein serine/threonine kinase activity) and four sub-ontologies that were significant among the sequentially decreasing gene group (group 2). In the transcripts that increased in expression from the mid-late to nonregressed late CL, followed by a decrease in functionally regressed late CL (group 3), one primary (catalytic activity) and two sub-ontologies were identified (supplemental Table 2).

Differentially expressed genes found by pairwise comparison of mid-late vs. functional late, or functional late vs. functionally regressed late stages were also analyzed for significant ontologies (supplemental Table 3). In the mid-late vs. functional-late pairwise comparison, differentially expressed transcripts that increased in expression outnumbered those that decreased in expression by a more than 2:1 ratio. For those transcripts that decreased in expression, there were two primary ontologies (oxidoreductase activity, cytoskeletal protein binding) and two sub-ontologies that were significant among these genes. Validation of microarray data

A total of 13 genes was selected for quantification of mRNA levels by Q-PCR to validate the microarray results. Because the late-stage CL were divided based on whether or not functional regression had occurred (serum levels of P4 at CL collection), genes encoding steroidogenic proteins were singled out for further analysis. There were five genes that met the criteria for differential expression from the mid-late through functionally regressed late stages whose gene products are involved in LH-stimulated steroidogenesis (P4 or estradiol) (Fig. 2A). Of these,
four were validated with regard to mRNA levels by Q-PCR (Fig. 2B). Statistically significant differences between groups were identical as determined by both microarray and Q-PCR methodologies for all four genes. With the exception of HSD3B2, mRNA levels for all of the differentially expressed genes related to LH-stimulated steroidogenesis did not change significantly from the mid-late to functional late stage but had a significant decrease in expression from the functional late to functionally regressed late CL groups as determined by microarray. For HSD3B2 gene expression, all three experimental groups were significantly (P < 0.05) different from one another.

Several differentially expressed genes encode proteins involved in cholesterol uptake, transport, and efflux. Six of these were selected for validation of microarray results by Q-PCR (Fig. 3). Q-PCR results were highly similar to the microarray findings in almost all cases. The primary exception was LDLR whose mRNA levels did not change from the mid-late to functional late stage as determined by microarray, whereas Q-PCR detected a significant (P < 0.05) 2-fold decrease from the mid-late to functional late CL. The LDLR and SCARB1 lipoprotein receptors both had their lowest mRNA levels in functionally regressed late CL (≥3- and 4-fold decreases, respectively, as determined by microarray and Q-PCR). In contrast, significant (P < 0.05) increases in mRNA levels were observed for ABCA1 and ABCG1, proteins that are involved in cholesterol efflux (30, 31), with their highest levels observed in functionally regressed late CL (≥ 4-fold total increases for both genes) (Fig. 3). In addition, APOL2 and APOL4 mRNA levels significantly (P < 0.05) increased from mid-late to functional late CL (≥2 and 3-fold increases, respectively).

Due to the known role of prostaglandins in initiating luteolysis in many nonprimate species, we searched for differentially expressed genes that encode proteins involved in prostaglandin biosynthesis, metabolism, and signaling. Two genes, HPGD and PTGES, were among the 681 differentially expressed transcripts from the mid-late through functionally regressed late stages. Moreover, by pairwise comparison, PTGFR also increased in expression between mid-late and functional-late stages, then decreased from functional to functionally regressed late stages. The mRNA levels of HPGD, PTGES, and PTGFR were quantified by Q-PCR (Fig. 4). Both microarray and Q-PCR detected a significant (P < 0.05) 3-fold or more decrease in HPGD mRNA from functional to functionally regressed late CL, whereas there was a significant (P < 0.05) 2-fold or more decrease in PTGES mRNA from the mid-late to functional-late CL. There was a significant (P < 0.05) 3-fold or more decrease in PTGFR mRNA from functional to functionally regressed late CL, as determined by both methodologies (Fig. 4).

**Corresponding protein levels of differentially expressed mRNAs**

The corresponding protein levels for each of five differentially expressed mRNAs were determined by Western blot analyses.
Among the steroidogenic proteins, levels of STAR, HSD3B2, and CYP19A1 were determined (Fig. 5). Concentrations of the mature form of STAR (30 kDa) were not different between mid-late and functional late CL; however, there was a significant ($P < 0.05$) 4.6-fold decrease in STAR in functionally regressed late CL. All experimental groups were significantly different ($P < 0.05$) from one another with regard to HSD3B2 protein. There was a 1.9-fold decrease from mid-late to functional late CL, with HSD3B2 levels being nearly nondetectable in functionally regressed late CL. There was no significant difference in CYP19A1 between mid-late and functional late CL, however, there was a significant ($P < 0.05$) 5.4-fold decrease in CYP19A1 levels in functionally regressed compared with functional-late CL (Fig. 5).

There were two bands (~25 and 33 kDa) detected by the anti-HPGD antibody that may represent two isoforms of HPGD (29 kDa predicted molecular mass) because both bands disappeared upon preabsorption of the antibody with its immunizing peptide (data not shown). Levels of either band did not change significantly across groups (Fig. 6). Levels of PTGES were significantly ($P < 0.05$) higher in mid-late CL than either functional or functionally regressed late CL (minimum 3.3-fold decrease).

**Discussion**

The current study details the generation of a database containing gene expression changes occurring immediately before and after the onset of functional regression in the rhesus macaque CL. Luteolysis is arguably the most poorly understood process associated with CL life span in primates. Functional regression appears to be the key event in this process because CG, which is essential for establishing pregnancy in primates, stimulates P4 synthesis via the LHCGR (8, 11), steroid ablation prevents CG-mediated rescue of the CL during simulated early pregnancy (22), and numerous luteotropic or antiluteolytic processes that may be modulated specifically by P4 have been reported (2). With regard to the latter, P4 suppresses expression of certain proteases (32), and inhibits immune-mediated luteolytic events (33). As a result of our initial analysis of differential gene expression throughout the normal luteal phase (23), it was clear that the most dynamic changes in gene expression were associated with the period encompassing functional and structural regression (late to very late-stage transition). Thus, because functional regression is likely the key event in luteolysis, it became apparent that a more-detailed analysis of gene expression profiles uniquely associated with this process was necessary.

As expected, dynamic changes in gene expression were observed throughout spontaneous functional regression of the macaque CL. There were three general patterns of expression found among differentially expressed genes occurring from the mid-late through functionally regressed late stages, including: 1) genes whose mRNA levels increased sequentially from the mid-late through the functionally regressed late stage; 2) transcripts that decreased sequentially from the mid-late through the functionally regressed late stage; and 3) genes whose mRNA levels increased from mid-late to nonregressed late CL, then decreased in functionally regressed late CL. We predict that transcripts increasing in expression from the mid-late to functional-late CL represent genes that encode pro-luteolytic proteins. On the other hand, transcripts that decrease in expression from the mid-late to functional late luteal phase are predicted to encode luteotropic proteins. On the other hand, transcripts that decrease in expression from the mid-late to functional late luteal phase are predicted to encode luteotropic proteins necessary for CL function.

Overlapping ontologies among differentially expressed genes revealed mechanisms or cellular processes that potentially facilitate luteolysis of the primate CL. The transcription factor activity ontology, as well as transcriptional repressor activity and transcriptional corepressor activity sub-ontologies were significant among genes that increased in expression (supplemental
These findings indicate that there may be a general suppression of transcription after functional regression of the primate CL. This is consistent with our previous microarray findings that there was more differential gene expression during the late to very late (CL collected a few days after functional regression occurred, d 18–19 after LH surge) stage transition than between all earlier stages of the luteal phase combined, with the majority of the genes in the late to very late stage transition exhibiting decreased mRNA levels (23). In addition, when performing pairwise comparisons, we found that the majority of differentially expressed transcripts increase in expression from the mid-late to functional late stages. In contrast, the majority of differentially expressed transcripts from the functional to functionally regressed late CL decreased in expression. This indicates that there is an initial increase in expression of what are most likely pro-luteolytic genes before the onset of functional regression, followed by a suppressive pattern of global gene expression after functional regression occurs. This is further evidence that functional regression is the key event in luteolysis because the predominant suppression of gene transcription from the late to very late stages that was noted in our previous study (23) may have been a consequence of the initial increase in expression of genes associated with transcriptional repression as observed in this study.

Another significant ontology among genes with increased levels of expression from the mid-late to late luteal phase (supplemental Table 2) included molecular adaptor activity (z score = 5.26). Genes in this ontology facilitate signaling from cell surface receptors by controlling receptor trafficking (e.g. Grb2-associated binding protein-1) (34) or by serving as important structural and functional constituents of large signaling complexes (e.g. tyrosine kinase receptors). For example, Grb2-associated binding protein-1 has been identified as a docking protein in epidermal growth factor and insulin receptor signaling (35), whereas chimerin 2 (36) and intersectin 2 (37) may serve as guanine nucleotide exchange factors that activate Ras-related guanosine 5′-triphosphatases (GTPases). Interestingly, the third primary ontology that was significant among genes increasing in expression from the mid-late to late luteal phase was GTP binding, which had a related sub-ontology of GTPase activity (z score = 4.09). Most of these genes are members of, or are related to, the RAS family, which are proteins that transduce signals from different tyrosine kinase activating receptors. Collectively, the increase in expression of genes associated with molecular adaptor and GTP binding ontologies may facilitate enhanced signaling from certain receptors that are necessary for luteolysis of the primate CL.

Among the three primary ontologies that were significant in the list of differentially expressed genes that decreased in expression from the mid-late to functionally regressed late CL (supplemental Table 2), oxidoreductase activity had the highest z score and the greatest total number of genes. Several of the genes in this ontology belong to the cytochrome P450 family. Two are associated with P4 and estradiol synthesis (HSD3B2 and CYP19A1), as well as biosynthesis of their cholesterol precursor (HMGCR, SLE). Thus, as expected, several genes associated with steroid or cholesterol biosynthesis decrease in expression during functional regression.

For the genes that increased in expression from the mid-late to functional late stages and then decreased in expression in the functionally regressed late stage, catalytic activity was the only significant primary ontology identified, with hydrolase and peptidase activity sub-ontologies also being noted (supplemental Table 2). It may not be surprising that an increase in expression of hydrolytic and proteolytic genes occurs before functional regression, however, the most-likely explanation of the role they may play in functional regression is not immediately clear. Because some of their gene products can be extracellularly located (e.g. ADAMTS14) (38, 39), there may be modification or degradation of cell surface receptors, extracellular ligands, or extracellular matrix constituents that are necessary for maintenance of CL function. Other genes in this group encode proteins that may be involved in immune-related processes that occur during luteolysis. For example, endoplasmic reticulum aminopeptidase 1 and
2 proteins act in a concerted mechanism to cleave peptides for subsequent presentation by major histocompatibility complex type-1 molecules (40). In any case, it seems logical that hydrolytic and proteolytic gene expression is consistent with a pro-luteolytic function.

When ontology analysis was performed on differentially expressed genes identified through pairwise comparisons, many ontologies were the same as those identified through the multiple comparison procedure, although they were not always due to differences in the ontologies were the same as identified through the multiple procedures validated the microarray data because only minor differences were noted (e.g. LDLR Q-PCR results, HPGD Western blot results). The process of luteolysis in primates is poorly understood. Therefore, further research to delineate how luteolysis occurs in the primate is needed, and this publicly accessible database (http://www.ncbi.nlm.nih.gov/geo/; accession no. GSE12807) can be a valuable resource for these investigations.

In summary, the current study provides a comprehensive analysis of gene expression across the entire macaque transcriptome during functional regression of the CL. Microarray results were compared with Q-PCR data, and the corresponding protein levels were determined by Western blot for selected genes. These procedures validated the microarray data because only minor differences were noted (e.g. LDLR Q-PCR results, HPGD Western blot results). The process of luteolysis in primates is poorly understood. Therefore, further research to delineate how luteolysis occurs in the primate is needed, and this publicly accessible database (http://www.ncbi.nlm.nih.gov/geo/; accession no. GSE12807) can be a valuable resource for these investigations.

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Address all correspondence and requests for reprints to: Jon D. Hennembold, Oregon Health and Science University, Oregon National Primate Research Center, 505 Northwest 185th Avenue, Beaverton, Oregon 97006. E-mail: hennemb@ohsu.edu.

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**FIG. 6.** Differential expression of proteins involved in prostaglandin E2 synthesis and metabolism. A, Western blots using samples pooled from CL collected at each stage. The bottom image is TUBB, which served as a loading control. B, Levels of HPGD and PTGES from individual CL (n = 4 per stage) were normalized to TUBB, and the resultant ratio was analyzed by ANOVA followed by comparison between groups using the Student-Newman-Keuls test. Columns with different letters are significantly different (P < 0.05). FL, Function late; FRL, functionally regressed late; ML, mid-late.