Antiproliferative Effects of Local Anesthetics on Mesenchymal Stem Cells

Potential Implications for Tumor Spreading and Wound Healing

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ABSTRACT

Background: Mesenchymal stem cells (MSC) are self-renewing clonal progenitor cells of nonhematopoietic tissues that exhibit a marked tropism to wounds and tumors. The authors’ studies aimed at exploring how local anesthetics would affect MSC biology.

Methods: Proliferation, colony formation, in vitro wound healing, and bone differentiation assays of culture-expanded bone-marrow-derived murine MSC were performed in the presence of increasing concentrations of lidocaine, ropivacaine, and bupivacaine. Cytotoxicity was monitored by measuring lactate dehydrogenase activity and phosphatidylserine exposure/propidium iodide staining (early apoptotic cells/necrotic cells). Measurements of mitochondrial function in intact and permeabilized cells, transcriptional changes, and changes in nuclear factor-κ-light-chain-enhancer of activated B cells signaling in MSC treated with ropivacaine were used to further characterize the biologic effects of local anesthetics on MSC.

Results: All local anesthetics reduced MSC proliferation at 100 μM, consistent with cell cycle delay or arrest at the G0/G1-S phase transition. They increased lactate dehydrogenase release and the number of annexin V-positive MSC but not necrotic MSC. Colony formation was decreased, differentiation into osteoblasts impaired, and in vitro wound healing delayed. Mitochondrial respiration and adenosine 5′-triphosphate concentrations were reduced. Microarray analysis revealed significant expression changes in lysosomal genes and genes controlling sterol metabolism, indicating an impaired phospholipid metabolism in the lysosome. Multiple transcriptional programs related to cell differentiation, tumorigenesis, and metastasis were negatively affected by ropivacaine.

Conclusions: The authors’ studies demonstrate that local anesthetics significantly affect important aspects of MSC biology. These experiments provide novel rationales for the perioperative use of local anesthetics in patients with cancer but also highlight the potentially detrimental effects of local anesthetics on wound healing.

What We Already Know about This Topic

- Mesenchymal stem cells are implicated in wound healing and tumor growth
- Local anesthetics have antiproliferative effects on many cell types, possibly including tumor cells, but their effects on mesenchymal stem cells are unknown

What This Article Tells Us That Is New

- Local anesthetics impaired proliferation, differentiation, and respiration and were cytotoxic to murine mesenchymal stem cells in vitro
- The possibility of beneficial antitumor effects and detrimental effects on wound healing in vivo requires additional study

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Mesenchymal stem cells (MSC) are self-renewing clonal progenitor cells of nonhematopoietic tissues that exhibit a marked tropism to wounds and tumors. A tumor is often regarded as a “nonhealing wound,” and vice versa, a wound can be regarded as “a healing tumor” because of the many similarities between tumor growth and tissue repair. MSC are recruited from the bloodstream to tumors, healing wounds, or sites of tissue injury by multiple growth factors and chemokines, where they differentiate into fibroblasts, pericytes, endothelial cells, and even terminally differentiated cells, such as osteoblasts, chondrocytes, astrocytes, neurons, and myocytes (“multilineage differentiation”). Engrafted at the sites of tissue damage, they secrete growth factors and cytokines (e.g., vascular endothelial growth factor, platelet-derived growth factor) that facilitate vasculogenesis and the healing process. Conversely, most experimental studies also show that MSC promote tumor growth. Coinjection of bone-marrow–derived MSC with green fluorescent protein-labeled breast cancer cells into immune-incompetent mice accelerates tumor growth. Likewise, coinjection of adult- and fetal-derived MSC with colon cancer cells into a mouse xenograft model leads to increased formation of highly vascularized tumors. Although some studies report proinflammatory and antiproliferative effects of MSC on tumor growth, probably by increased mobilization of macrophages and granulocytes, MSC are known to secrete proangiogenic factors, such as vascular endothelial growth factor, fibroblast-derived growth factor, platelet-derived growth factor, and stromal cell-derived factor-1, which potentially facilitate endothelial and smooth muscle cell proliferation in tumors. MSC also secrete chemokine (C-C motif) ligand 5, which was shown to enhance the metastatic potential of breast cancer cells.

Local anesthetics are commonly used in the perioperative setting for pain treatment to reversibly block the conductance in neurons (regional anesthetics, nerve blocks, wound infiltration). If overdosed, they exert detrimental toxic effects mainly on neural and cardiac tissues resulting in life-threatening seizures and respiratory and cardiac arrest. Reports on their cytotoxicity also revealed adverse effects on mitochondrial respiration resulting in marked oxidative stress. Other studies indicate a dose-dependent inhibition of proliferation of fibroblasts and tenocytes at concentrations as low as 10 μM. These observations raise concerns that direct administration (e.g., using wound catheters) of local anesthetics at even low concentrations could weaken the wound and delay its closure. Conversely, there is emerging evidence that local anesthetics applied in the perioperative setting are capable of preventing tumor spreading during cancer surgery. So far, it remains elusive whether local anesthetics affect the biology of MSC, key players in tissue repair and tumor growth. Therefore, we hypothesized that lidocaine, bupivacaine, and ropivacaine would inhibit the proliferation of MSC in a dose-dependent manner and set out to unravel the underlying mechanisms. The expected inhibition of MSC by local anesthetics could indeed represent an important mechanism by which local anesthetics applied in the perioperative period might help prevent perioperative metastasis and improve long-term survival of patients undergoing cancer surgery. Conversely, inhibition of MSC proliferation by prolonged application of local anesthetics potentially could delay wound closure and promote wound dehiscence.

Materials and Methods
The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (Publication No. 85-23, revised 1996), and the experimental protocol used in this investigation was approved by the University of Alberta Animal Policy and Welfare Committee (Edmonton, Alberta, Canada).

Supplies and Chemicals
All chemicals were purchased from Sigma (Oakville, Ontario, Canada) unless otherwise stated. Dulbecco modified Eagle’s medium, fetal bovine serum, penicillin-streptomycin, trypsin-EDTA solution, and Dulbecco phosphate buffered saline were obtained from Invitrogen (Burlington, Ontario, Canada).

MSC Isolation and Expansion
Mesenchymal stem cells were isolated from femurs and tibias of C57BL6/J mice 8–10 weeks old. Marrow was extruded by inserting a 26.5-gauge needle into the shaft of the bone and flushing it with complete cell culture media (Dulbecco modified Eagle’s medium supplemented with 20% fetal bovine serum and 1% penicillin-streptomycin). The aspirate was resuspended in complete media and distributed into T75 cell culture flasks. The flasks were incubated at 37°C in a humidified atmosphere of 5% CO₂-95% air in a cell culture incubator. After 24 h, nonadherent cells were removed, fresh media was added, and the adherent cells were allowed to reach 80% confluence, before splitting. Passage 3 cells were used for immunophenotypical characterization and for the osteogenic differentiation assay. Passage 7–15 (P7–P15) cells were used for all other experiments.

Characterization of MSC
Fluorescence-activated cell sorting (FACS) of cell surface antigens was performed to characterize the immunophenotype of MSC, in accordance with the minimal criteria for definition of MSC. MSC were collected, counted on a Coulter counter, and cryopreserved. Characterization of MSC was performed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, California). The following Abs were used for immunophenotypical characterization and for the osteogenic differentiation assay: anti-CD105 (clone MJ7/18; Biolegend, Burlington, Ontario, Canada), anti-CD117 (clone c-kit; BD Biosciences, San Jose, California), anti-CD34 (clone 5G10; BD Biosciences, San Jose, California), anti-CD44 (clone BVD4-4G8; Biolegend), anti-CD45 (clone 2B8; Biolegend), anti-CD90 (clone 5E10; Biolegend), anti-CD31 (clone PAC-1; BD Biosciences, San Jose, California), anti-CD14 (clone HCD14; Biolegend), and anti-CD43 (clone 2F1; Biolegend). The following Abs were used in osteogenic differentiation assay: anti-CD73 (clone AD7; BD Biosciences, San Jose, California), anti-CD90 (clone 5E10; Biolegend), anti-CD105 (clone MJ7/18; Biolegend), anti-CD117 (clone c-kit; BD Biosciences, San Jose, California), and anti-CD14 (clone HCD14; Biolegend).
Mesenchymal stem cells were treated with TNF-α (20 ng/ml) and exposed to increasing concentrations of local anesthetics for 60 min. Cells were collected, washed, resuspended in FACS buffer, and incubated for 30 min with a R-phycocerythin–conjugated monoclonal ICAM-1 antibody (clone 3E2) or with isotype-matched control antibody (BD Biosciences) in the dark at 4°C. Fluorescence signals of scatter-gated cells were measured using FACSCanto II flow cytometer and FACS-Diva software.

Expression of Cell Cycle-related Proteins

The expression of the cell cycle regulatory components p16/INK4a, p27/Kip1, and proliferating cell nuclear antigen was investigated by Western blotting in MSC exposed to ropivacaine for 24 h. Cells were collected, washed twice with ice-cold phosphate buffered saline, and centrifuged at 6,000 g for 10 min, and the pellets were snap-frozen in liquid N2.
Western blotting, cell pellets were thawed in lysis buffer (20 mM Tris, pH 7.4; 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, Triton X-100 (1%), 2.5 mM sodium pyrophosphate, protease, and phosphatase inhibitor cocktails). Cell lysates were homogenized and centrifuged at 12,000g for 15 min at 4°C, and protein concentrations were determined with the Bradford assay (Bio-Rad, Hercules, CA). Proteins were separated on SDS-polyacrylamide (12%) gels and electrophoretically transferred onto a nitrocellulose membrane (Bio-Rad, Mississauga, Ontario, Canada). Membranes were probed overnight at 4°C with the following primary antibodies: rabbit anti-p16 INK (SAB4500072; Sigma), rabbit anti-p27 KIP1 (SAB4500068; Sigma), antiproliferating cell nuclear antigen (clone PC10, 2586 Cell Signaling Technology; distributed by New England Biolabs Ltd., Pickering, Ontario, Canada), α-tubulin (loading control; T6074; Sigma). After incubation with antimicroglobulin G horseradish-peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for 1.5 h at room temperature, protein bands were quantified using ImageJ software.§§

**Immunoblotting and Electrophoretic Mobility Shift Assay in TNFα-treated Cells**

Mesenchymal stem cells were treated for 60 min with 20 ng/ml TNFα in the presence or absence of ropivacaine 100 μM and collected and processed for Western blotting as described. Primary antibodies against nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (IkB) were rabbit anti-IκB-α (ab7217; Abcam, Cambridge, MA) and rabbit antiphiphospho-IκB-α (ab12135; Abcam). Electrophoretic mobility shift assays were performed using the nuclear factor κ-light-chain- enhancer of activated B-cells (NF-κB) p65 LightShift Chemiluminescent electrophoretic mobility shift assay kit (Product No. 89859; Thermo Scientific, Rockford, IL). Nuclear extracts were prepared from frozen cell pellets using the NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Product No. 78833; Thermo Scientific).

**High-resolution Respirometry in Intact and Permeabilized Cells**

The respiratory capacity of the mitochondrial electron transport chain in control and ropivacaine (100 μM)-treated MSC after 24 h of incubation was measured with a high-resolution respirometry (Oxygraph-2K; Oorostr, Innsbruck, Austria) at 37°C. Analysis of the coupling states in intact cells was performed with membrane-permeant inhibitors or uncouplers (see figure, Supplemental Digital Content 1, http://links.lww.com/ALN/A820, for a detailed protocol). To evaluate the site(s) of inhibition by ropivacaine, we measured phosphorylation rates in digitonin-permeabilized cells in the presence of various mitochondrial complex-specific substrates and/or inhibitors (see figure, Supplemental Digital Content 2, for a detailed protocol, http://links.lww.com/ALN/A821). At the end of every run, cell suspensions were collected and stored at −80°C for citrate synthase activity measurements.

**Determination of Adenosine 5′-triphosphate (ATP) Concentrations**

Adenosine 5′-triphosphate concentrations were measured in control and ropivacaine (100 μM)-treated MSC after 24 h of incubation using the ATP bioluminescent somatic cell assay kit (Sigma). A subset of samples was also treated with either iodoacetate (200 μM; inhibitor of glycolysis) or antimycin A (10 μM, complex III inhibitor) during the last hour of the incubation period. These additional experiments were performed to assess the contribution of glycolytically produced ATP to total ATP in MSC. Data were normalized to citrate synthase activity.

**Citrate Synthase Activity**

To relate the observed respiration rates to mitochondrial content, the activity of the mitochondrial matrix marker enzyme citrate synthase was measured at 412 nm by monitoring the formation of thionitrobenzoate, the product of reaction between 5,5′-dithiobis-2-nitrobenzoate (a chromogen) with the thiol group of free coenzyme A that is produced in the formation of citrate.16 The reaction was initiated by the addition of 0.5 mM oxaloacetate in the presence of 0.3 mM acetyl-coenzyme A, and the rate of absorbance change was monitored for 2 min.

**Measurements of Reactive Oxygen Species (ROS)**

Cellular production of hydrogen peroxide in response to ropivacaine was measured by loading cells with 2′,7′-dichlorodihydrofluorescein diacetate (20 μM; diluted in the medium from a 10-mM stock solution in dimethyl sulfoxide) for 45 min in the dark. During the last 30 min, ropivacaine (100, 250, and 500 μM) or antimycin A (2.5 μM, positive control) was added to the plates. Cells not loaded with the fluorescent dye served as negative controls. Cells were harvested, resuspended in FACS buffer, and their fluorescence signals collected using FACSCanto II flow cytometer and FACSDiva software.

**Microarray Analysis**

Control and ropivacaine (100 μM)-treated MSC were collected after a 24 h-incubation and processed for total RNA isolation using the Qiagen RNeasy MiniKit (QIAGEN Inc., Toronto, Ontario, Canada) according to the manufacturer’s instructions. RNA samples were processed for microarray analysis (Affymetrix Mouse Exon 1.0 ST Arrays; Affymetrix, Santa Clara, CA) in accordance with the minimum information about a microarray experiment (MIAME) guidelines.17
Data are available at the Gene Expression Omnibus database under the series number GSE31827. Gene set enrichment analysis was performed to assess alterations in global gene expression in response to ropivacaine.18,19 Microarray results were confirmed by real-time polymerase chain reaction assays (the primers used and the validation data are presented in the table, Supplemental Digital Content 3, http://links.lww.com/ALN/A822).

### Statistical Analysis

Values are given as mean ± SD for the indicated number of independent observations (n). The significance of differences in variables among groups was determined by Student t test (two groups) or by analysis-of-variance (ANOVA) followed by the Holm-Sidak method for post hoc analysis or by nonparametric methods (Kruskal-Wallis test) depending on the underlying data distribution. Proliferation data were analyzed using two-way ANOVA followed by the Holm-Sidak method for post hoc multiple comparisons. Wound healing assay data were analyzed using two-way repeated measures ANOVA followed by the Student–Newman–Keuls test. Differences are considered significant if P < 0.05. SigmaStat (version 3.5; Systat Software, Inc., Chicago, IL) was used for the analyses.

### Results

The immunophenotypical characterization by flow cytometry revealed that MSC were uniformly positive for stem cell antigen-1 (97.4%) and CD105 (endoglin; 96.7%) but negative for the hematopoietic-endothelial antigens CD34 (0.4%), CD45 (0.3%), and c-kit (CD117; 0.2%). MSC also expressed the hyaluronan receptor CD44 (8.5%).

### Local Anesthetics Dose-dependently Exert Antiproliferative Effects, Increase Markers of Cellular Injury, Delay In Vitro Wound Healing, and Impair Osteogenic Differentiation in MSC

For clarity, ropivacaine data are presented. Comparative results obtained with lidocaine, bupivacaine, and ropivacaine are depicted in figures 1–4 of Supplemental Digital Content 4 (http://links.lww.com/ALN/A823). Ropivacaine inhibited cell proliferation at concentrations ≥100 μM (fig. 1A). The population doubling time in untreated cultures was 39.4 h...
but increased to 92.7 h in cultures treated with 100 μM ropivacaine. Inhibition of cell growth was accompanied with lactate dehydrogenase release (fig. 1B), a marker of cytotoxicity and increased plasma membrane permeability. A dose-dependent impairment of colony formation (fig. 1C) and an increase in annexin V binding to phosphatidylserine (marker of early apoptosis) (fig. 2A) were observed.

Cell cycle analysis after 24 h of exposure to ropivacaine revealed a higher percentage of MSC in G0/G1 phase and a lower percentage in the S phase (fig. 2B), consistent with cell cycle arrest. Ropivacaine up-regulated the expression of the cell cycle-regulatory proteins p16INK4a, p27Kip1, and proliferative cell nuclear antigen in whole cell lysates after exposure to increasing concentrations (100, 250 μM) of ropivacaine (C). *Significantly different from CTL. #Significantly different from 100 μM. Data are mean (SD); n = 4 (A) and n = 6 (B and C). CTL = control without treatment; PCNA = proliferative cell nuclear antigen; ROPI = ropivacaine.

Fig. 2. Apoptosis markers and cell cycle. Annexin V (early apoptotic) and annexin V-propidium iodide (PI; late apoptotic) positive cells after exposure to increasing concentrations (100, 250 μM) of ropivacaine for 24 h (A). Cell cycle phases (G0/G1, S, G2/M) of mesenchymal stem cells exposed to 100 and 250 μM ropivacaine for 24 h indicative of cell cycle arrest (B). Expression patterns of the G1 phase regulatory proteins p16INK4a, p27Kip1, and proliferative cell nuclear antigen in whole cell lysates after exposure to increasing concentrations (100, 250 μM) of ropivacaine (C). *Significantly different from CTL. #Significantly different from 100 μM. Data are mean (SD); n = 4 (A) and n = 6 (B and C). CTL = control without treatment; PCNA = proliferative cell nuclear antigen; ROPI = ropivacaine.

Ropivacaine Reduces the Expression of ICAM-1, a Key Surface Receptor in MSC Migration and Differentiation, via the IκB–NF-κB Signaling Pathway

Because previous studies demonstrated the importance of ICAM-1 in MSC migration and differentiation,20,21 we hypothesized that local anesthetics would decrease ICAM-1 expression in the presence of TNFα. Ropivacaine markedly decreased TNFα-induced ICAM-1 expression in MSC in a concentration-dependent manner (fig. 4A). IκB phosphorylation and NF-κB translocation to nuclei subsequently were determined in MSC exposed to ropivacaine. Ropivacaine inhibited TNFα-induced IκB phosphorylation (fig. 4B) and abolished translocation of transcriptional factor NF-κB to nuclei (fig. 4C).
Ropivacaine Inhibits Mitochondrial Respiration Depleting Cellular ATP Content and Imposes Oxidative Stress on MSC

Mitochondrial oxygen consumption was measured in intact and permeabilized MSC, and all data were normalized to citrate synthase activity. No difference in citrate synthase activity between the control and ropivacaine-treated cells was observed (data not shown). Intact MSC treated with 100 μM ropivacaine exhibited a significant reduction (~28%) in oxygen consumption while respiring on endogenous substrates (fig. 5A). The treated MSC also exhibited a significant reduction (~25%) in maximal mitochondrial oxidative capacity as assessed by carbonyl cyanide-p-trifluoromethoxyphenylhydrazone-induced oxygen consumption. However, the leak respiration rate ( oligomycin-insensitive fraction of respiration ) was unchanged. To determine the site(s) of inhibition by ropivacaine ( i.e. , to isolate the flux going through specific respiratory complexes), we used a multiple substrate-inhibitor combination protocol in permeabilized MSC. Our experiments show marked reductions in all examined complexes ( i.e. , complex I, complex II, and complex IV; fig. 5B). Glutamate-malate–driven flux through complex I was decreased by 34% in ropivacaine-treated MSC compared with untreated cells. Complex II-dependent respiration was even more impaired (40% reduction), whereas complex IV-driven flux was reduced by 30%. Ropivacaine induced a small but significant dose-dependent increase in the production of ROS (fig. 5C). Subsequent experiments showed that 100 μM ropivacaine exposure for 24 h also reduced cellular ATP content by ~20% compared with untreated MSC (fig. 5D). To determine whether the cellular ATP depletion was entirely attributable to inhibition of mitochondrial respiration, ATP content of MSC was measured after ropivacaine exposure in the presence of iodoacetate, an inhibitor of glycolysis, or antimycin A, a complex III inhibitor (fig. 5D). These experiments demonstrate that MSC can produce ATP through glycolysis in the presence of oxygen (Warburg effect), and the reduction in ATP concentrations in ropivacaine-treated cells is indeed attributable to dysfunctional mitochondria. To test whether inhibition of mitochondrial respiration by ropivacaine would be causally related to their antiproliferative action, MSC were concomitantly exposed to ropivacaine and the antioxidant N-acetylcysteine. Antimycin A, an inhibitor of the respiratory chain, dose-dependently delayed MSC proliferation in a manner similar to that of ropivacaine and served as positive control. Treatment with the antioxidant N-acetylcysteine did not reverse the effect of ropivacaine, implying that mechanisms other than ROS...
Transcriptional Profiling UnCOVERS a Lysosomal Storage Disorder but also Reveals the Anticancer Potential of Ropivacaine Elicited in MSC

Ropivacaine treatment (100 μM, 24 h) induced significant transcriptional changes in MSC compared with untreated cells. Among the top up-regulated transcripts are genes related to cholesterol metabolism (e.g., lanosterol synthase, mevalonate [diphospho]decarboxylase), the lysosome (e.g., β-galactosidase, lysosomal ATPase), cell cycle control (e.g., dipeptidyl-peptidase 2, G0/G1 switch gene 2), and stress response (e.g., metallothioneins). However, ropivacaine treatment repressed genes related to differentiation processes (e.g., pleiotrophin, asporin, transcription factor Sp7/osterix, and osteoglycin; see figure, Supplemental Digital Content 7, http://links.lww.com/ALN/A826). Gene set enrichment analysis clearly confirmed that amphiphilic local anesthetics have detrimental effects on membranes. Ropivacaine significantly increased the metabolism of lipids and cholesterol, essential components of mammalian cell membranes, but also up-regulated lysosomal processes, consistent with an increase phospholipid turnover (table 1; figure, Supplemental Digital Content 8, http://links.lww.com/ALN/A827). Ropivacaine reduced the expression of chemokines (figure, Supplemental Digital Content 8, http://links.lww.com/ALN/A827) and of pathways related to angiogenesis and metastasis formation (fig. 6, table 1, and table 2). The analysis also uncovered important perturbations of transcriptional developmental programs caused by ropivacaine treatment (table 2).

Discussion

Our experiments were motivated by recent findings that the perioperative use of local anesthetics appears to improve long-term survival in cancer patients, which could be caused by anti-proliferative cytostatic effects of local anesthetics on tumor cells.10 In a retrospective analysis of patients undergoing surgery for breast cancer, the use of a paravertebral nerve block combined with general anesthesia was associated with a better cancer-free survival.22 A number of additional studies could con-
Table 1. Representative Induced and Repressed Pathways in Mesenchymal Stem Cells Treated with 100 μM Ropivacaine

<table>
<thead>
<tr>
<th>GSEA Pathway</th>
<th>Brief Description</th>
<th>NES*</th>
<th>Nominal P Value</th>
<th>FDR q-Value†</th>
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<tr>
<td>REACTOME_CHOLESTEROL_BIOSYNTHESIS</td>
<td>Genes involved in cholesterol biosynthesis</td>
<td>2.224</td>
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<td>REACTOME_STEROID_METABOLISM</td>
<td>Genes involved in steroid metabolism</td>
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<td>LIAN_LIPA_TARGETS_6 M</td>
<td>Genes up-regulated at 6 months of age in lungs from LIPA§ knockout mice</td>
<td>1.883</td>
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<td>0.019</td>
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<td>KEGG_LYSOSOME</td>
<td>Lysosomes are membrane-delimited organelles in animal cells serving as the cell's main digestive compartment</td>
<td>1.745</td>
<td>0.000</td>
<td>0.066</td>
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<tr>
<td>REACTOME_METABOLISM_OF_LIPIDS_AND_LIPOPROTEINS</td>
<td>Genes involved in the metabolism of lipids and lipoproteins</td>
<td>1.542</td>
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<td>KEGG_PEROXISOME</td>
<td>Genes involved in the catabolism of very long chain fatty acids</td>
<td>1.625</td>
<td>0.009</td>
<td>0.616</td>
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<td>RUTELLA_RESPONSE_TO_HGF_UP</td>
<td>Genes up-regulated by hepatocyte growth factor in peripheral blood monocytes</td>
<td>1.623</td>
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<td>RAMALHO_STEMNESS_DN</td>
<td>Genes depleted in embryonic, neural, and hematopoietic stem cells</td>
<td>1.499</td>
<td>0.021</td>
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<td>MARKEY_RB1_CHRONIC_LOF_DN</td>
<td>Genes down-regulated in MEF cells (embryonic fibroblasts) isolated from RB1§ knockout mice</td>
<td>1.796</td>
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<td>0.153</td>
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<td>GERY_CEBP_TARGETS</td>
<td>Genes changed in embryonic fibroblasts by expression of one or more of CCAAT-enhancer binding proteins</td>
<td>1.450</td>
<td>0.010</td>
<td>0.913</td>
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<td>GAUSSMANN_MLL_AF4_FUSION_TARGETS_G_DN</td>
<td>Down-regulated genes from set G: specific to cells expressing both MLL/AF4§ and AF4-MLL§ fusion genes (“leukemic” fusion genes)</td>
<td>1.834</td>
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<td>0.000</td>
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<td>GAUSSMANN_MLL_AF4_FUSION_TARGETS_E_UP</td>
<td>Up-regulated genes from set E: specific signature shared by cells expressing either MLL-AF4§ or AF4-MLL§ fusion proteins alone and those expressing both fusion proteins</td>
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<td>0.000</td>
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<td>REACTOME_PEPTIDE_LIGAND_BINDING_RECEPTORS</td>
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<td>REACTOME_GPCR_LIGAND_BINDING</td>
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<td>REACTOME_CHEMOKINE_RECEPTORS_BIND_CHEMOKINES</td>
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<td>IZADPANAH_STEM_CELL_ADIPOSE_VS_BONE_DN</td>
<td>Genes down-regulated in adipose tissue mesenchymal stem cells vs. bone marrow mesenchymal stem cells</td>
<td>−1.658</td>
<td>0.002</td>
<td>0.172</td>
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</table>

(Continued)
GSEA Pathway                                | Brief Description                                                                 | NES* | Nominal P Value | FDR q-Value† |
--- | --- | --- | --- | --- | --- |
VERRECCHIA_EARLY_RESPONSE_TO_TGFβ1        | Extracellular matrix protein-related genes up-regulated within 30 min in dermal fibroblasts after addition of TGFβ1 | −1.638 | 0.008 | 0.624 |
REACTOME_SIGNALING_BY_PDGF                | Genes involved in signaling by platelet-derived growth factor | 1.638  | 0.007 | 0.098 |
KEGG_HEDGEHOG_SIGNALING_PATHWAY          | Hedgehog proteins regulate morphogenesis of a variety of tissues and organs and control stem cell proliferation in adult tissues | −1.468 | 0.039 | 0.261 |

* Positive = induction or up-regulation by ropivacaine 100 μM; negative = repression or down-regulation by ropivacaine 100 μM.
† Estimated probability that a gene set with a given NES represents a false-positive finding.
‡ LIPA (gene ID = 3,988) encodes lipase A, the lysosomal acid lipase (also known as cholesteryl ester hydrolase). This enzyme functions in the lysosome to catalyze the hydrolysis of cholesteryl esters and triglycerides. LIPA-knockout mice represent a model of lysosomal acid lipase deficiency. § RB1 (gene ID = 5,925) encodes retinoblastoma-associated protein, which is a negative regulator of the cell cycle and was the first tumor suppressor gene found. The encoded protein also stabilizes constitutive heterochromatin to maintain the overall chromatin structure. Defects in this gene are a cause of childhood cancer retinoblastoma, bladder cancer, and osteogenic sarcoma. || MLL (gene ID = 4,297) encodes histone-lysine N-methyltransferase, also called myeloid-lymphoid or mixed-lineage leukemia protein. # AF4 (gene ID = 4,299) encodes AF4/FMR2 family member 1, also called ALL1-fused gene from chromosome 4 protein. AF4 is associated with acute leukemias.

FDR = false discovery rate; GSEA = gene set enrichment analysis; MEF = mouse embryonic fibroblasts; NES = normalized enrichment score; TGFβ1 = transforming growth factor β1.

Mesenchymal Stem Cells and Local Anesthetics

Table 1. (Continued)

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experiments do not support ROS formation as the single cause of the antiproliferative actions. Rather, our transcriptional analysis points to a number of additional mechanisms perhaps underlying ropivacaine-induced cell cycle inhibition. Our microarray analysis also showed up-regulation of genes controlled by the transcription factor sterol regulatory element-binding protein-1a, which is known to cause G₁ cell cycle arrest through accumulation of cyclin-dependent kinase inhibitors p16, p21, and p27,39 and up-regulation of the cell cycle inhibitory G₀G₁ switch gene 2. In our experiments, ropivacaine-treated cells did not show uncoupling, but uncoupling was shown previously for bupivacaine.34 Local anesthetics also were reported to directly inhibit F₁F₀ ATP synthase and to decrease depolarize the mitochondrial potential. Although mitochondrial dysfunction appears to be the predominant cause of cell cycle arrest and antiproliferative action in our experiments, we cannot entirely rule out that changes in lipid composition of mitochondrial and other membranes, as evidenced in our transcriptional analysis, may have contributed, at least on the longer-term (i.e., after 24 h) to mitochondrial dysfunction. In fact, local anesthetics similar to other cationic amphiphilic drugs, such as tetracyclines and amiodarone, induce steatosis and phospholipidosis, characterized by intracellular phospholipid and cholesterol-triglyceride accumulation interfering with vital cellular functions.42

Fig. 6. Genes containing the binding consensus sequence for the transcription factor ZEB1 (zinc finger E-box-binding homeobox 1, also called transcription factor 8) in their promoters are negatively affected by ropivacaine treatment (100 μM) for 24 h in cells. Because of space limitations, only the first 60 significantly regulated genes (of a total of 106) are depicted. Red indicates induction; green indicates repression of gene expression.
<table>
<thead>
<tr>
<th>GSEA Pathway</th>
<th>Brief Description</th>
<th>NES*</th>
<th>Nominal P Value</th>
<th>FDR q-Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCANNTGAY_V$SREBP1_01</td>
<td>Genes containing the motif TCANNTGAY, which matches annotation for SREBF1 (sterol regulatory element binding transcription factor 1)</td>
<td>1.451</td>
<td>0.003</td>
<td>0.102</td>
</tr>
<tr>
<td>V$HNF1_C</td>
<td>Genes containing the motif DGTTAATKAWTNACCAM, which matches annotation for hepatic nuclear factor (HNF1)</td>
<td>-1.642</td>
<td>0.000</td>
<td>0.919</td>
</tr>
<tr>
<td>TGGTTGY_V$HNF3_Q6</td>
<td>Genes containing the motif TGGTTGY, which matches annotation for FOXA1 (forkhead box A1)</td>
<td>-1.461</td>
<td>0.000</td>
<td>0.555</td>
</tr>
<tr>
<td>CAGGTA_V$AREB6_01</td>
<td>Genes containing the motif CAGGTA, which matches annotation for ZEB1 (AREB6/TCF8; zinc finger E-box-binding homeobox 1)</td>
<td>-1.433</td>
<td>0.000</td>
<td>0.447</td>
</tr>
<tr>
<td>V$CIZ_01</td>
<td>Genes containing the motif SAAAAANNN, which matches annotation for ZNF384 (zinc finger protein 384)</td>
<td>-1.485</td>
<td>0.007</td>
<td>0.542</td>
</tr>
<tr>
<td>V$AP2REP_01</td>
<td>Genes containing the motif CAGTGGG, which matches annotation for KLF12 (Kruppel-like factor 12)</td>
<td>-1.435</td>
<td>0.008</td>
<td>0.825</td>
</tr>
<tr>
<td>V$GATA4_Q3</td>
<td>Genes containing the motif AGATADMAGGGA, which matches annotation for GATA4 (GATA binding protein 4)</td>
<td>-1.439</td>
<td>0.010</td>
<td>0.704</td>
</tr>
<tr>
<td>V$SRF_Q6</td>
<td>Genes containing the motif GNCCAWATAWGGMN, which matches annotation for SRF (serum response factor; c-fos serum response element-binding TF)</td>
<td>-1.342</td>
<td>0.014</td>
<td>0.528</td>
</tr>
<tr>
<td>V$NF1_Q6_01</td>
<td>Genes containing the motif NTGGNNNNNNGCCAANN, which matches annotation for NF1 (neurofibromin 1; neurofibromatosis, von Recklinghausen disease, Watson disease)</td>
<td>-1.378</td>
<td>0.015</td>
<td>0.471</td>
</tr>
<tr>
<td>V$SOX9_B1</td>
<td>Genes containing the motif NNNNAACAAATRGN, which matches annotation for SOX9</td>
<td>-1.423</td>
<td>0.015</td>
<td>0.536</td>
</tr>
<tr>
<td>V$CRX_Q4</td>
<td>Genes containing the motif YNNNTAACCYCMN, which matches annotation for CRX (cone-rod homeobox)</td>
<td>-1.332</td>
<td>0.020</td>
<td>0.466</td>
</tr>
<tr>
<td>V$CART1_01</td>
<td>Genes containing the motif NNNTAAATTNCATTANCN, which matches annotation for CART1 (cartilage paired-class homeoprotein 1)</td>
<td>-1.351</td>
<td>0.021</td>
<td>0.527</td>
</tr>
<tr>
<td>V$AP4_Q6</td>
<td>Genes containing the motif CWGAGCTGGQN, which matches annotation for TFAP4 (transcription factor AP-4; activating enhancer binding protein 4)</td>
<td>-1.401</td>
<td>0.021</td>
<td>0.433</td>
</tr>
</tbody>
</table>

(Continued)
Colonization, because tumor cells prefer sites of injury and healing for growth and proliferation. Interestingly, it was shown that even very low subanesthetic concentrations of bupivacaine can become cytotoxic if applied over an extended time. Moreover, in our in vitro experiments, we used 20% serum to create optimal conditions for MSC to grow and proliferate. However, because proliferation is a balance between promoting and inhibiting growth stimuli, it is possible that under in vivo conditions with less favorable conditions and a functional tumor-inhibiting immune system, much lower concentrations of local anesthetics (i.e., in the nanomole range), may be sufficient to inhibit or kill tumor cells. Accordingly, Martinsson reports that a reduction in serum concentration from 10 to 1% increases the sensitivity of cultured HT-29 colon adenocarcinoma cells to ropivacaine-induced inhibition of proliferation by 50%. Nevertheless, it is possible that the putative improved outcome in cancer patients receiving local anesthetics and/or regional anesthesia is attributable to the concomitant pain relief leading to a reduced consumption of morphine, which has been reported to be proangiogenic. Clearly, our studies need additional in vivo validation.

**Considerations on the Use of Local Anesthetics against Tumor Growth and Metastasis Formation in Surgical Patients**

The role of MSC in tumor growth may be particularly important in the context of surgery, where tissue damage caused by surgery evokes a massive surge of these cells from the bone marrow. Most recent in vivo studies strongly support the tumor growth-promoting actions of MSC, although not all bone marrow-derived MSC may promote tumor progression equally. MSC promote tumor growth and metastasis formation in mul-

<table>
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</tr>
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<tbody>
<tr>
<td>V$MEF2_02</td>
<td>Genes containing motif KCTAWAAATAGM (related to GenBank X68505; H. sapiens mRNA for myocyte-specific enhancer factor 2)</td>
<td>−1.374</td>
<td>0.023</td>
<td>0.433</td>
</tr>
<tr>
<td>V$FXR_IR1_Q6</td>
<td>Genes containing the motif GGGTBAATRACCCY, which matches annotation for RXRA (retinoid X receptor, α)</td>
<td>−1.441</td>
<td>0.032</td>
<td>0.834</td>
</tr>
<tr>
<td>V$AP4_Q5</td>
<td>Genes containing the motif VDCAGCTGNNN, which matches annotation for TFAP4 (transcription factor AP-4; activating enhancer binding protein 4)</td>
<td>−1.296</td>
<td>0.041</td>
<td>0.448</td>
</tr>
<tr>
<td>V$TFIIA_Q6</td>
<td>Genes containing the motif TMTRWRAGGRSS, which matches annotation for GTF2A1 (general transcription factor IIA, 1)</td>
<td>−1.288</td>
<td>0.05</td>
<td>0.516</td>
</tr>
</tbody>
</table>

The pathways in this group collect genes with promoter regions (i.e., [-2kb,2kb] around transcription start site) containing transcription factor binding consensus sequences.

* Positive = induction or up-regulation by ropivacaine 100 μM; negative = repression or down-regulation by ropivacaine 100 μM.
† Estimated probability that a gene set with a given NES represents a false-positive finding.
FDR = false discovery rate; GSEA = gene set enrichment analysis; NES = normalized enrichment score.
tiple ways, including antiapoptotic proliferative effects, immuno-
us suppression, drug resistance, paracrine secretion of growth
factors and chemokines, and "epithelial-to-mesenchymal
transition." In addition, MSC have a propensity to homeing toward tumor cells and are prone to malignant
self-transformation because of chromosomal instability. Our unbiased microarray screen now reveals for the first time
that ropivacaine markedly down-regulates transcripts related to
G-protein coupled receptors, chemokines, and growth factor
signaling in MSC, consistent with antiproliferative antiinflammatory and cytostatic actions. Ropivacaine up-
regulates transcripts such as RB1, a negative regulator of the
cell cycle, known to be suppressed in retinoblastoma, bladder
cancer, and sarcoma. Our comprehensive analysis also shows that ropivacaine suppresses multiple gene sets with promoter
regions containing transcription factor consensus sequences
associated with stemness and/or cell differentiation. The iden-
tification of large groups of genes harboring common
transcription factor binding sites (table 2) is essential for
understanding the regulatory modules that control stem cell
processes such as differentiation or metabolism. However,
the false-discovery rates are rather high. Likely reasons for this
may be the limited number of samples per group (n = 4) and/or
the noise inherent to the expression data of these particular gene
sets. Irrespective of the underlying reasons, the results clearly
point to the necessity to further validate the findings. Among
the most prominent consensus sequences were -CAGGTA-
matching with transcription factor 8, also called ZEB1 (zinc
finger E-box-binding homeobox 1), and -NTGGN-
NNNNGCCAANN- matching with neurofibromin 1.
Transcription factor 8 or ZEB1 inactivity promotes tumor-
igenicity and metastasis by angiogenesis, whereas lack of
neurofibromin 1 enhances cell growth by enhancing signal
transducer and activator of transcription-3. Interestingly,
an MSC-like phenotype is the hallmark of tumor
aggressiveness in human primary glioblastomas. In this
study, genes that were down-regulated by ropivacaine,
such as collagen type III α1, transforming growth factor-β
induced, or tenasin C (see figure, Supplemental Digital Con-
tent 8, http://links.lww.com/ALN/A827), were highly overex-
pressed in aggressive glioblastoma tumors and strong predictors
of survival.

What Do Our Results Suggest with Respect to the Direct
Application of Local Anesthetics to Wounds?
Mesenchymal stem cells form an essential component of the
cellular wound healing process, which consists of cell migra-
tion and proliferation, deposition of extracellular matrix, vascu-
logenesis, and matrix metalloproteinase-mediated tissue remodel-
ing. Using an excisional wound splitting model in mice, Wu et al.
showed that injection of bone marrow derived MSC
around the wound markedly accelerated wound healing and
closing in normal and diabetic mice. These authors also ob-
erved increased formation of angiopeitin-1 and endothelial cell
tube formation after MSC injection, indicating that MSC par-
ticipate in revascularization, a critical step in tissue repair. Red-
uced wound breaking strength and impaired healing were re-
ported in rat models of acute wound repair after exposure to
local anesthetics. A recent study using a mouse model of cu-
taneous wound healing was unable to demonstrate adverse ef-
ects of lidocaine and bupivacaine, but the local anesthetics were
applied only once over 3 days as bolus and not as continuous
infusion.

Study Limitations
The local anesthetics used in our study are widely administered
in perioperative medicine. The question of whether ester local,
as opposed to amide local, anesthetics or general anesthetics
(namely volatile anesthetics and propofol) would have similar or
opposite effects on MSC is important and should be tested in
future experiments. We recognize that the results of our exper-
iments were obtained through in vitro primary cell cultures of
MSC. Although it is inappropriate to use our data for extrapol-
atation to the clinical environment, they provide valuable novel
information about mechanisms underlying the putative anti-
cancer and tissue repair inhibiting effects of local anesthetics.
Clearly, future in vivo experimental and clinical studies will be
necessary to clarify the roles of local anesthetics and MSC in
perioperative tumor spreading and tissue repair.

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Fleming Brothers’ Crudoform Liniment of Chloroform and Ether

In 1888 Pittsburgh’s Fleming Brothers Company featured products on an advertising calendar (above) titled The Dream of Life. Targeting female “rheumatics,” the handout included seasonal poetry highlighting painful changes that weather might bring to arthritic joints: “First budding Spring appears, / Next Summer’s heat, / Then Autumn’s fruits, / Then Winter’s cold and sleet.” Rheumatism was characterized in this calendar as “That dire disease, whose ruthless power / Withers the beauty’s transient flower.” To avoid withering their beauty or suffering needlessly from arthritis, ladies were encouraged to liberally rub Crudoform liniment on afflicted areas. (This Fleming Brothers liniment contained a mixture of chloroform and ether.) An inset page (lower right) from the calendar does not clarify whether the monkey (pulling the cat’s tail) symbolized Rheumatism’s taunting of the afflicted or represented the new-found agility of arthritic patients relieved by Crudoform. . . . (Copyright © the American Society of Anesthesiologists, Inc.)

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