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Requirement of N-Myristoyltransferase 1 in the Development of Monocytic Lineage

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N-myristoyltransferase (NMT) exists in two isoforms, NMT1 and NMT2, that catalyze myristoylation of various proteins crucial in signal transduction, cellular transformation, and oncogenesis. We have recently demonstrated that NMT1 is essential for the early development of mouse embryo. In this report, we have demonstrated that an invariant consequence of NMT1 knock out is defective myelopoiesis. Suppressed macrophage colony forming units were observed in M-CSF-stimulated bone marrow cells from heterozygous (+/−) Nmt1-deficient mice. Homozygous (−/−) Nmt1-deficient mouse embryonic stem cells resulted in drastic reduction of macrophages when stimulated to differentiate by M-CSF. Furthermore, to understand the requirement of NMT1 in the monocytic differentiation we investigated the role of NMT, pp60c-Src (NMT substrate) and heat shock cognate protein 70 (inhibitor of NMT), during PMA-induced differentiation of U937 cells. Src kinase activity and protein expression increased during the differentiation process along with regulation of NMT activity by hsc70. NMT1 knock down in PMA treated U937 cells showed defective monocytic differentiation. We report in this study novel observation that regulated total NMT activity and NMT1 is essential for proper monocytic differentiation of the mouse bone marrow cells. The Journal of Immunology, 2008, 180: 1019–1028.

Hematopoiesis is a finely orchestrated developmental cascade leading to the generation of distinct lineages culminating into the mature blood cell types. The family of cells constituting mononuclear phagocytic system arises from hematopoietic progenitors in bone marrow developing and differentiating into monocytes from promonocytes. Monocytes migrate into tissue after entering into circulation to become mature macrophages. M-CSF (1) is the major cytokine responsible for the production of monocytes and macrophages in adult mice among other cytokines/lymphokines such as GM-CSF, IL3, and so on (2, 3). c-Src is the cellular homologue of v-Src, the transforming gene of Rous Sarcoma Virus (4) and the founding member of a group of nonreceptor tyrosine kinases (5). c-Src plays a central role in numerous signaling pathways impacting cellular proliferation, transformation, differentiation, motility, and survival (6–8). Overexpression and/or activation of c-Src is linked genetically and biochemically to the development of several human cancers, especially those of the colon and breast (9, 10). Although there is limited knowledge on the role of c-Src in monocytic differentiation, it is definitely an important player in the cellular differentiation signaling (11). The specific subcellular localization of pp60c-Src manifests different functions. For instance, if it is localized at the plasma membrane, it is involved in the mitogenic signaling via growth factors. When localized in cytoplasm or a perinuclear region, it regulates protein trafficking (12). Similarly c-Src localizes to both focal adhesions and adherence junctions where it functions in cellular adhesion and migration.

The membrane association is dependent on the myristoylation of pp60c-Src. N-myristoylation is a cotranslational modification of proteins (13) in which myristate (a 14-carbon fatty acid) is covalently attached to the NH2 terminus of various cellular, viral, and oncoproteins (14–17). Cellular myristoylated proteins have diverse biological functions in signal transduction and oncogenesis (14, 15, 18, 19). Tyrosine kinases of the Src family are among the most extensively studied myristoylated proteins.

Myristoylation of proteins is catalyzed by the ubiquitously distributed eukaryotic enzyme N-myristoyltransferase (NMT) (15–17). Earlier, we reported for the first time in a rat model that NMT is more active in colonic epithelial neoplasm than in the corresponding normal appearing colonic tissue, and that an increase in NMT activity appears at an early stage in colonic carcinogenesis (20).

NMT activity can be regulated by N-myristoyltransferase Inhibitor Protein 71 (NIP71) (21); remarkably, we have recently shown that NIP71 is homologous to heat shock cognate protein 70 (hsc70) (22). Recently, we demonstrated that NMT1 is essential for the early development of the mouse and is the main enzyme in early embryonic development (23). Because homozygous (−/−) Nmt1-deficient mouse did not survive, we were interested in investigating whether NMT1 plays crucial role in mouse hematopoiesis. Suppressed macrophage CFU’s were observed in heterozygous (+/−)

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3 Abbreviations used in this paper: NMT, N-myristoyltransferase; NIP71, N-myristoyltransferase inhibitor protein 71; hsc70, heat shock cognate protein 70; ES, embryonic stem cell; WT, wild type; siRNA, small interfering RNA; BMC, bone marrow derived macrophages; MMC, bone marrow cell; M-CSF, mouse M-CSF.

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**Materials and Methods**

In brief, 1,10-[3H] myristic acid (39.3 Ci/mM) was purchased from PerkinElmer. *Pseudomonas* acyl CoA synthetase was purchased from ICN Biochemicals. The polyvinylidene difluoride membrane was purchased from BioRad Laboratories. NMT-1 mAb and HRP-conjugated goat anti-mouse Ab was obtained from BD Biosciences. c-Src mAb was purchased from Santa Cruz Biotechnology and monoclonal anti-hsc70 was bought from Cedarlane Laboratories. Recombinant mouse M-CSF was purchased from R&D Systems. Cell culture reagents, RPMI 1640, and FBS were purchased from Cederlane Laboratories. Recombinant mouse M-CSF was purchased from Santa Cruz Biotechnology and monoclonal anti-hsc70 was bought from BioRad Laboratories. NMT-1 mAb and HRP-conjugated goat anti-mouse Ab was obtained from BD Biosciences. c-Src mAb was purchased from Santa Cruz Biotechnology and monoclonal anti-hsc70 was bought from Cedarlane Laboratories. Recombinant mouse M-CSF was purchased from R&D Systems. Cell culture reagents, RPMI 1640, and FBS were purchased from Cederlane Laboratories. Recombinant mouse M-CSF was purchased from Santa Cruz Biotechnology and monoclonal anti-hsc70 was bought from Cedarlane Laboratories. Recombinant mouse M-CSF was purchased from R&D Systems. Cell culture reagents, RPMI 1640, and FBS were purchased from Cederlane Laboratories.

**Bone marrow derived macrophages (BMDM)**

Animal care and use were in accordance with the guidelines of the Canadian Council on Animal Care. Macrophages were derived from bone marrow of C57BL/6 mice. Bone marrow cells (BMC) were obtained from the femurs of mice. BMC were obtained from the femoral shafts by flushing it with PBS. BMC were then agitated gently to prepare a single cell suspension and were washed subsequently three times in PBS. BMC were then incubated for 4 h in a humidified air and 5% CO₂ at 37°C to allow resident bone marrow macrophages to adhere. After 4 h, nonadherent cells were collected and allowed to grow in the presence or absence of 10 ng/ml recombinant mouse M-CSF (mMCSF). After washing, the cells that remained adhered to the plastic culture plates were BMDM. Localization of c-Src in BMDM was studied by immunofluorescence analysis.

**Bone marrow colony-forming assay**

Colony-forming ability of BMC was assayed using MethoCult medium (Stem Cell Technologies) according to the manufacturer’s protocol. In brief, BMC (1 × 10⁵ cells/ml) were suspended in a MethoCult containing FBS, BSA, and 2-ME in presence of mouse GM-CSF, G-CSF, or M-CSF used as myeloid-lineage specific hematopoietic growth factor at an optimal dose of 10 ng/ml. The mixture was gently vortexed and 1 ml each was plated in 35 mm plastic tissue culture petri-dishes and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. Bone marrow colonies were counted after 14 days of incubation. An aggregate of >25 cells were counted as a single colony. Colonies were considered as a single colony. Colonies with >80% of the macrophages or granulocytes were designated as CFU-M or CFU-G, respectively. Mixed-type colonies containing 10 mM DTT, 1 mM PMSF, and 1% protease inhibitor mixture (Sigma-Aldrich) was used for the cell lysis.

**RNA interference**

Small interfering RNA (siRNA) duplexes designed against human NMT1 and hsc70 were obtained from Dharmacon. Scrambled siRNA from Dharmacon was used as the control siRNA. siRNAs were transfected into cells by DharmaFect I. U937 cells (10⁵) were treated with 10 ng/ml PMA and were allowed to incubate in a humidified air and 5% CO₂ at 37°C for 4 h for each set of experiments. Once the cells were adhered, lipid-mediated transfection was performed. We mixed 10⁵ cells, 50 μl siRNA (2 μM), and 4 μl DharmaFECT 1 in 146 μl of OptiMEM medium according to procedures provided by Dharmacon. The transfected cells were placed in a 35 mm plate containing 0.8 ml of cell culture medium or chambered slides. The cells were either harvested or fixed at 24, 48, 72, and 120 h after siRNA treatment.

**Cell culture**

Promonocytic U937 cell line was obtained from American Type Culture Collection. Cells were grown in RPMI 1640 supplemented with 10% FCS and 1% antibiotic solution (Invitrogen Life Technologies) in a humidified air and 5% CO₂ at 37°C. Cells were harvested at indicated time points upon treatment with PMA (10 ng/ml). RIPA buffer (50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) was used as myeloid-lineage specific hematopoietic growth factor at an optimal dose of 10 ng/ml. The mixture was gently vortexed and 1 ml each was plated in 35 mm plastic tissue culture petri-dishes and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. Bone marrow colonies were counted after 14 days of incubation. An aggregate of >25 cells were counted as a single colony. Colonies were considered as a single colony. Colonies with >80% of the macrophages or granulocytes were designated as CFU-M or CFU-G, respectively. Mixed-type colonies containing 10 mM DTT, 1 mM PMSF, and 1% protease inhibitor mixture (Sigma-Aldrich) was used for the cell lysis.
with >40% of either macrophages or granulocytes were designated as CFU-GM type colonies.

Nmt1-deficient mice

A mouse ES cell line (XE400, strain 129/Ola) with an inserational mutation in Nmt1 was created in a gene-trapping program, BayGenomics (baygenomics.ucsf.edu). Details are provided in Ref. 23.

Isolation of homozygous Nmt1-deficient ES cells

Nmt1<sup>−/−</sup> ES cells were isolated as described earlier (23). In brief, Nmt1<sup>−/−</sup> ES cells were grown for 24 h in ES cell medium in a 100-mm gelatin-coated petri dish. Single colonies were picked and grown under G418 selection pressure (12.0 mg/ml). Cell lines were genotyped by both Southern blot and PCR.

Analysis of macrophage differentiation of ES cells

Nmt1<sup>−/−</sup> ES or WT ES cells were cultured as described (baygenomics.ucsf.edu). In brief, ES cells were plated at a concentration of 1 × 10<sup>4</sup> cells on a 25 cm<sup>2</sup> tissue culture plate precoated with 0.1% gelatin and grown in glasgow minimum essential medium GMEM (Sigma-Aldrich) supplemented with 2 mM glutamine (Invitrogen Life Technologies), 1 mM sodium pyruvate, 1 mM nonessential amino acids, 10% (v/v) FBS, a 1/1000 dilution of 2-ME of stock solution, and 10 mM Mg-BSA for 4 days at 37°C in 5% CO<sub>2</sub> for differentiation to macrophages. Cells were collected in PBS and stained with AlexaFluor 488 anti-F4/80 (eBioscience) and analyzed using FACS.

 Src kinase activity

An in vitro c-Src kinase assay was performed according to the reported method (25). In brief, U937 lysate containing 500 μg total cellular protein was incubated with excess pp60<sup>−src</sup> specific mAb at 1 h at 4°C. Immune complexes were collected by the addition of 30 μl of 30% protein G-Sepharose slurry, incubated for 30 min at 4°C, and followed by centrifugation at 10000 × g for 1 min at 4°C. Immunoprecipitated pellets were resuspended in 40 μl of a kinase reaction buffer containing 0.25 M Hepes (pH 7.4), 25 mM MgCl<sub>2</sub>, 0.75 M NaCl, 1 μl [γ-<sup>32</sup>P]ATP, 10 μM ATP, 300 μM Sr octopeptide (AEEIEYGFEEAKKKK) (26), 200 μM sodium orthovanadate, and 4 mg/ml p-nitrophenol phosphate for 15 min at 30°C. Reactions were terminated by the addition of 25 μl of 50% (v/v) acetic acid. Aliquots from each reaction were spotted onto P81 phosphocellulose paper squares, which were subsequently washed three times in 0.5% (v/v) phosphoric acid, dried, and counted in a scintillation counter; two independent experiments were performed.

N-myristoyltransferase assay

N-Myristoyltransferase activity was assayed as described earlier (27). In brief, [<sup>1</sup>H]Myristoyl-CoA was synthesized according to the literature (27). The reaction mixture contained 40 mM Tris-HCl (pH 7.4), 0.1 mM EGTA, 10 mM MgCl<sub>2</sub>, 5 mM ATP, 1 mM LiCoA, 1 mM [H]Myristic acid (7.5 μCi), and 0.3 unit/ml Pseudomonas acyl-CoA synthetase in a total volume of 300 μl. The reaction was conducted for 30 min at 30°C. The conversion to [<sup>1</sup>H]Myristoyl-CoA was greater than 95%. The assay mixture contained 40 mM Tris-HCl (pH 7.4), 0.5 mM EGTA, 0.45 mM 2-ME, 1% Triton X-100, peptide substrate (500 μM), and NMT in a total volume of 25 μl. The transferase reaction was initiated by the addition of freshly generated [<sup>1</sup>H]Myristoyl-CoA and was incubated at 30°C for 30 min. The reaction was terminated by spotting 15 μl aliquots of incubation mixture onto P81 phosphocellulose paper discs and drying under a stream of warm air. The P81 phosphocellulose paper discs were washed in two changes of 40 mM Tris-HCl (pH 7.3) for 30 min. One unit of NMT activity was expressed as 1 pmol of myristoyl peptide formed per min.

Northern analysis

U937 cells were treated with PMA for the indicated time periods and then harvested. RNA was isolated as described earlier (28). Equal amounts (15 μg) of total cellular RNA were fractionated in 1% denaturing formaldehyde-agarose gels. RNA was transferred to Gene Screen nylon membranes (DuPont) by capillary elution, UV cross-linked, and then hybridized with cDNA probes specific for c-Src or β-actin labeled with [α-<sup>35</sup>S]dCTP using an oligolabeling kit (Pharmacia). Autoradiography was performed at ~80°C using an intensifier screen (Kodak).

Western analysis

Western blot analysis was performed essentially as described previously (29). Samples were electrophoresed on a 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane. Immoblot was developed using polyclonal anti-NIP71 Ab or mAb against NMT-1, pp60<sup>−src</sup>, or hsc70.

Immunofluorescence analysis

For immunofluorescence analysis, U937 cells treated with PMA for different time points and grown in chambered slides. This was followed by washing thrice with 1 ml PBS containing 1 mM sodium azide and thereafter fixed with 0.5 ml 4% paraformaldehyde (pH 7.4) for 30 min at room temperature. After washing as above, subsequent steps were conducted in dark. The cells were incubated with 5 μg/ml fluorescent-tagged secondary Ab for 2 h at room temperature and washed thrice as before. Coverslips were mounted on the slides using anti-fade mounting medium. Cells were visualized under a Zeiss LSM410 confocal microscope at 488 nm and 594 nm excitation. For colocalization, experiments two relevant primary Abs (monoclonal anti-c-Src and polyclonal anti-mannosidase II or polyclonal anti-NIP71) raised in different species and a mixture of red and green fluorescent-tagged species-specific secondary Abs were used.

Immunoprecipitation

Cells were lysed in RIPA buffer containing 10 mM DTT, 1 mM PMSE, and 1% protease inhibitor mixture (Sigma-Aldrich). Src and hsc70 were precipitated using an Immunoprecipitation Starter Pack (protein G Sepharose 4 fast flow; Amersham Biosciences) following the manufacturer’s protocols.

Other methods

Proteins were estimated by the Bradford method (30). SigmaPlot software (SYSTAT) was used for the statistical analysis.

Results

NMT1 is essential for monocytic differentiation

Heterozygous (+/−) Nmt1-deficient mice carrying a β-galactosidase marker gene were generated as described elsewhere (23). Bone marrow from WT C57BL/6 mice or heterozygous (+/−) Nmt1-deficient mice obtained from femur was allowed to grow in presence or absence of mCMCSF (10 ng/ml) for 7 days. As depicted in Fig. 2A, Wright-Geimsa-stained BMDM from heterozygous (+/−) Nmt1-deficient mouse are not the same as that of BDMMDWT mice. Total number of BMDM obtained from heterozygous (+/−) Nmt1-deficient mouse were almost one-fourth of BDMMDWT. In addition, BDMMDWT displayed more abundant cytoplasm with cytoplasmic projections and presence of few cytoplasmic granules. These features are not present in BMDM from heterozygous (+/−) Nmt1-deficient mice (Fig. 2A). Furthermore, to understand the role of NMT1 in the myelopoesis, we determined the colony forming ability of the bone marrow from WT and heterozygous (+/−) Nmt1-deficient mice. Bone marrow cells obtained from WT and heterozygous (+/−) Nmt1-deficient mice were cultured for 14 days in the colony assay semisolid medium in the presence or absence of GM-CSF, M-CSF, or G-CSF. The total number of colonies and the differential number of individual CFU of CFU-G, CFU-GM, CFU-M, and CFU-G were enumerated as described in the Materials and Methods, the results are shown in Fig. 2B. Incubation of the bone marrow cells of WT mice consistently resulted in a higher number of total CFU compared with that of Nmt1 (+/−) mice. The number of CFU-M remains significantly higher in the bone marrow cells of WT mice irrespective of being incubated in the presence of GM-CSF, M-CSF, or G-CSF compared with that of Nmt1 (+/−) mice. The number of CFU-G and CFU-GM were observed to remain comparable in the bone marrow cells of WT and (+/−) mice upon incubation with G-MCF or M-CSF. However, the number of CFU-GM and CFU-G was significantly higher in the bone marrow cells of WT mice compared with that of Nmt1 (+/−) mice when incubated in the presence of G-CSF. Taken together, the result of bone...
Marrow cells forming assay indicates a partially diminished ability of Nmt1 (+/-) mice to differentiate in myeloid lineage.

We isolated WT and homozygous (+/-) Nmt1-deficient ES cells and cultured them on a feeder-independent system as described elsewhere (23). ES cells were cultured in presence of M-CSF for 96 h, and the macrophage population was determined by the expression of F4/80 surface marker by flow cytometry. Interestingly, expression of AlexaFluor 488-conjugated F4/80 gated on the majority of cells was observed in 17.0% of cells in the WT ES cells (Fig. 2C) whereas, in the homozygous (−/-) Nmt1-deficient ES cells, the expression of F4/80 was detected in only 1.4% cells.

There was no appreciable difference in the mean channel intensity of the Ag detected by F4/80, further validating that NMT1 is essential for the proper monocytic differentiation.

**NMT activity and its regulation by N-myristoyltransferase inhibitor protein**

From the above observation, it was clear that NMT is essential for the differentiation of monocyte/macrophages. We further investigated whether there is any alteration of its activity during the differentiation process. We determined NMT activity at different time points of the BMDM maturation.
stage of macrophage differentiation, reaching a maximum at 72 h and then decreased for the remaining time course. BMDM from heterozygous (+/-) Nmt1-deficient mice showed similar NMT activity profile, although the activity was almost half compared with BMDM from WT mouse BM (Fig. 3A) except at the 72 h time point, where the NMT activity of BMDM from WT and heterozygous (+/-) Nmt1-deficient mice showed almost similar activity. This is due to the presence of half the catalytic amount of protein in BMDM from heterozygous (+/-) Nmt1-deficient mice. Parabolic enzymatic activity could be due to the differential NMT expression or may be due to the regulation of activity by its inhibitor protein. Therefore, we examined the expression of NMT in BMDMWT at different time points (Fig. 4). NMT expression increased slightly during the maturation of BMDMWT. In addition, we also studied the expression of NMT inhibitor protein hsc70. Interestingly, hsc70 increased with time (Fig. 4).

To further investigate the mechanistic implication of NMT in monocytic differentiation, we selected the U937 promonocytic cell line as a model in vitro system, as it can be induced to differentiate along the monocyte/macrophage cell lineage by PMA (24). Following PMA treatment, U937 cells become adherent and, over the course of several days, differentiate into a monocyte/macrophage phenotype. Differentiation of promonocytic U937 cells into macrophages is well documented by the changes in cell morphology, including development of pseudopodia and significant levels of NF-κB (11, 31). We examined NMT activity at similar time points to determine whether the NMT activity profile in U937 cells follows the activity trend of the BMDM maturation process. Interestingly, the NMT activity increased during the initial period of differentiation, reaching a maximum at 48 h, but then dramatically fell over the remaining time course (Fig. 5A). To determine

![Image](https://www.jimmunol.org/)

**FIGURE 3.** NMT activity profile during the maturation of BMDM. A, Bone marrow cells were treated with mM-CSF for different time periods to obtain BMDM. NMT activity in BMDM from the bone marrow of wild type C57BL/6 mice (□) or heterozygous (+/-) Nmt1-deficient mice (■) was measured for indicated time points using pp60°-Src derived peptide substrate. Values are mean of at least three independent experiments ± SD. B, Localization of c-Src and hsc70 at indicated time points described below during the differentiation course of BMDM WT (WT C57BL/6 mice) or BMDM Nmt (+/-) (heterozygous (+/-) Nmt1-deficient mice) in the presence of mMCSF (10 ng/ml). I; 12 h, II; 24 h, III; 48 h, IV; 72 h, V; 96 h, VI; 120 h, VII; 144 h, VIII; 168 h, IX; 192 h.

![Image](https://www.jimmunol.org/)

**FIGURE 4.** Expression of NMT and hsc70 during the differentiation of BMC. BMDM were obtained from WT C57BL/6 mice as described under Materials and Methods. Cells were lysed in RIPA buffer and subjected to 10% SDS-PAGE and Western blot analysis was conducted using monoclonal anti-NMT1 Ab, polyclonal anti-hsc70 Ab, or monoclonal anti-β actin Ab (1/1000 dilution, used as the loading control indicator).
whether this activity profile was due to differential protein expression, we measured NMT levels by Western analysis. As shown in Fig. 5B, NMT expressions increased with the differentiation process, reaching a maximum at 72 h and remaining elevated thereafter. The above results suggest that the observed decrease in NMT activity after 48 h may be due to the induction of NMT inhibitor(s). We therefore examined the expression of NIP71/hsc70 during the differentiation process. As shown in Fig. 4C, there was a dramatic induction of NIP71 beginning at 72 h that correlated closely with the observed decrease in NMT activity. To further establish that hsc70 in vivo regulates NMT activity, we knocked down hsc70 using siRNA in the PMA-treated U937 cells. We observed that NMT activity was restored upon knockdown of hsc70 (Fig. 5D). Above findings clearly indicate that regulated NMT activity is invariably associated with monocytic/macrophage differentiation.

c-Src induction during U937 differentiation

c-Src is rendered membrane bound and activated upon myristoylation. Its induction during PMA-induced differentiation of U937 had previously been examined (32). To explore the possible relationship between NMT and c-Src during differentiation process, we examined c-Src expression and its kinase activity. As shown in Fig. 6A, c-Src mRNA was dramatically induced during this process beginning at 12 h, reaching a maximum at 48 h and remaining

**FIGURE 5.** NMT activity and protein expression in U937 cells treated with PMA for different time periods. A, NMT activity was measured for indicated time points using pp60c-Src derived peptide substrate. Values are mean of at least three independent experiments ± SD. Proteins (25 μg) of U937 cells treated with PMA for indicated time points were subjected to 10% SDS-PAGE, transferred onto polyvinylidene difluoride membrane and probed with (B) monoclonal anti-NMT1 Ab (1/250 dilution) or (C) polyclonal anti-NIP71 Ab (1/1000 dilution). D, NMT activity in the lysates of PMA treated U937 cells at indicated time points after transfection with (light gray) Scramble or (dark gray) hsc70 siRNA in the presence of c-AMP dependent protein kinase derived peptide.
elevated throughout the remainder of the time course. This induction of c-Src mRNA was closely paralleled by an increase in both pp60^{c-Src}/H11002 and expression and associated kinase activity (Fig. 6, B and C). These results demonstrate that c-Src is induced during this differentiation process. c-Src is usually associated with cytoplasmic or plasma membrane fractions and myristoylation is essential for its localization.

**Translocation of c-Src, NMT, and hsc70**

Because NMT activity is severely curtailed during the differentiation process, we decided to examine the cellular localization of c-Src during the process. As shown in Fig. 7, c-Src is predominantly cytoplasmic at early time points. However, at around 48 h, it becomes increasingly associated with a perinuclear region reminiscent of the Golgi apparatus (Fig. 9, panel I, 48 h). In later time points, c-Src is predominantly associated with this structure (Fig. 7). To determine whether this region was indeed the Golgi, we carried our additional immunofluorescence studies with Abs specific to mannosidase II, a common marker for the Golgi (Fig. 7). As shown in Fig. 7, both mannosidase II and c-Src colocalize, confirming c-Src localizes to the Golgi during differentiation of U937 cells. NMT is predominantly cytosolic. However, there have been reports of multiple forms and membrane-associated NMT (33–35). NMT remained cytoplasmic during the differentiation process (Fig. 9A, NMT1, panel I). In general, hsc70 is cytoplasmic,

**FIGURE 6.** c-Src mRNA, protein expression and kinase activity in U937 cells treated with PMA. A, Northern blot of total RNA from U937 cells probed specifically for c-Src. B, Western blot analysis of U937 cell lysates from treated with PMA for indicated time points using monoclonal anti-c-Src Ab (1/1000 dilution). C, In vitro kinase assay of c-Src immunoprecipitated from U937 cell lysates. c-Src kinase activity expressed as pmol of phosphate incorporated into synthetic Src optimal peptide AEEEY GEPEAKKKK per min per mg of total cellular protein. Values are mean of at least three independent experiments ± SD.

**FIGURE 7.** Localization of c-Src, NMT, and hsc70 during PMA induced differentiation of U937 cells. Immunofluorescence studies were conducted to localize c-Src and hsc70 at 6 h and 96 h after PMA treatment. For showing translocation of c-Src and hsc70 into Golgi; c-Src or hsc70 and mannosidase II were colocalized.

**FIGURE 8.** Evidence for c-Src and hsc70 protein-protein interaction. U937 cells were treated with PMA for 120 h. c-Src and hsc70 from two different lysates were immunoprecipitated using monoclonal anti-c-Src and anti-hsc70 Abs. Immunoprecipitated complexes were subjected to 10% SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and were probed with monoclonal anti-hsc70 Ab. Lane 1: hsc70 immunoprecipitated complex; Lane 2: c-Src immunoprecipitated complex; Lane 3: purified hsc70 (hc- H chain of Ab, lc- L chain of Ab).
which under various stresses is translocated into the nucleus (36, 37). It is interesting to note that during the differentiation process, hsc70 also appeared to translocate from the cytoplasm to the Golgi apparatus, as shown in Figs. 7 and 9. Thus, given hsc70’s known chaperone activity, it is highly likely that the trafficking of c-Src from cytoplasm to Golgi apparatus is aided by hsc70.

Src-hsc70 interaction

The colocalization of Src and hsc70 to the Golgi apparatus during U937 differentiation is a unique observation and suggests that c-Src and hsc70 might interact directly. To test this hypothesis, we immunoprecipitated Src and hsc70 from the cell lysate of U937 cells treated with PMA for 120 h (Fig. 8) using anti-Src and anti-hsc70 mAbs. The immunoprecipitated complexes were electrophoresed on a 10% SDS gel and Western analysis was conducted. A 70-kDa band was visualized when probed with anti-hsc70 mAb. This suggests c-Src interaction with hsc70 during the differentiation process of U937 cells into monocytic cells. Control experiments were run using preimmune serum for the specificity of the interaction. No bands specific for c-Src and hsc70 were observed (data not shown) supporting the interactions above.

NMT1 is essential for the differentiation of U937 cells

The above results correlating the translocation of c-Src and hsc70 into the Golgi apparatus together with the regulated NMT activity during the differentiation of the U937 cells strongly suggests that NMT plays a crucial role during this differentiation process. We, therefore, specifically knocked down NMT1 or NMT2 using siRNA in PMA treated U937 cells. We selected 24, 48, 72, and 120 h time points. Nonadherent U937 cells start adhering at 1 h after treatment with PMA. Gradually, in the course of time, these cells spread and take a flattened morphology typically of macrophages. After 24 h, cells remain small and circular, which over a period of time grows in size and flattens into macrophage. Cells transfected with scrambled siRNA (control) showed a normal differentiation process (Fig. 9A, panel I). Cells transfected with NMT1 siRNA for 120 h resulted in increased cell death, and the morphology of the cells did not change and were same as that of 24 h (Fig. 9A, panel II). Interestingly, hsc70 and c-Src remained cytoplasmic in the majority of the cells (Fig. 9A, panel II, hsc70 and c-Src panel). c-Src and hsc70 translocated to the Golgi apparatus in the cells transfected with scramble siRNA (Fig. 9A, panel I, hsc70 and c-Src panel) and Golgi apparatus was identified using mannosidase II (Fig. 9B). In the cells transfected with scrambled siRNA, c-Src and hsc70 translocated into the Golgi apparatus (Fig. 9A, panel I, hsc70 and panel 120 h), whereas NMT1 knock down render c-Src and hsc70 cytoplasmic (Fig. 9A, panel II, hsc70 and panel 120 h). To further substantiate that hsc70 and c-Src translocate into the Golgi apparatus during differentiation of U937 cells, in cells transfected with scrambled siRNA, c-Src and mannosidase II colocalized at two different time points, 24 and 72 h (Fig. 9B, panel I, merge), whereas, transfection with NMT1 siRNA rendered c-Src cytoplasmic (Fig. 9B, panel II, merge). NMT2, hsc70, or c-Src knockdown did not alter the differentiation process and their translocation during the course of differentiation in U937 cells (A. Shrivastav and R. K. Sharma, unpublished data).

Discussion

In this study, we have examined the role of NMT during monocytic differentiation. BMDM from heterozygous (+/-) Nmt1-deficient mice are defective when compared with BMDM from WT mice. More matured BMDM were obtained from WT mice. It is evident from the presence of macrophages with more abundant cytoplasm with projections and presence of granules (Fig. 2A). More studies are required to follow the difference in the maturation of BMDM from WT and heterozygous (+/-) Nmt1-deficient mice in terms of their phagocytic ability and macrophage marker presentation. In addition, the number of macrophage-type colonies derived from bone marrow cells of heterozygous (+/-) Nmt1-deficient mice were less frequent than those derived from WT mice.
We demonstrated that ablation of NMT1 affects the proper monocytic differentiation and reduced the expression of F4/80 molecule in homozygous (−/−) Nmt1-deficient ES cells. The F4/80 is the first documented surface marker for the appearance of monocyte/macrophage cells in the mouse and detects an Ag that is expressed on maturing monocyte/macrophages (38, 39). During the course of differentiation of bone marrow cells and U937 cells into macrophage, NMT activity followed similar trends in both systems. Although the activity was almost half in the BMDM of heterozygous (+/−) Nmt1-deficient mice as compared with BMDM of WT mice except 72 h (Fig. 3A), where both have similar activity. c-Src is a physiological substrate of NMT and is involved in various vital cellular pathways. We demonstrated c-Src mRNA is induced at 12 h, reaching maximum at 48 h during PMA-induced differentiation of U937. Protein expression of c-Src and kinase activity also increases in concordance with mRNA expression. Our results are in agreement with a report suggesting activation of c-Src during differentiation of U937, indicating that c-Src activation is a normal physiological process associated with myeloid differentiation (32).

Concomitant induction of c-Src and kinase activity with mRNA induction suggests involvement of c-Src in the differentiation process. NMT activity increased in the initial stages of differentiation of BMDM and U937 cells, reaching maximum at 72 and 48 h, respectively, thereafter decreased considerably throughout the remaining of the differentiation process due to the induction of NMT inhibitor(s). We have previously described a membrane-bound potent inhibitor protein of NMT having a mass of 71 kDa, referred to as NIP71 (21). Recently, we demonstrated that NIP71 is homologous to hsc70 (22). Induction of NIP beginning at 72 h suggests that NMT activity is regulated by NIP71/hsc70. The molecular chaperone hsc70 binds polypeptides in an ATP-dependent cycle that is involved in various cellular functions such as protein folding, sorting, and transportation (40). The importance of hsc70 molecular chaperons stems from its function of folding and translocation of proteins, because several diseases arise from defects in these processes (41, 42). There are reports that hsc70 is involved in differentiation of promonocytic U937 cells. An example is the exogenous addition of hsc70 up-regulated monocyte/macrophage markers CD11c and CD23 × 20–35% (43). In the case of HL-60, promyelocytic leukemia differentiation, there were no significant changes in hsc70 levels (44). In addition, hsc70 neither had a stimulatory effect on the maturation of dendritic cells nor did it effect the differentiation of monocytes into dendritic cells (45). Induction of hsc70 after 48 h coupled with the observed decrease in NMT activity is consistent with our hypothesis that hsc70 functionally attenuates NMT activity. In addition, knockdown of hsc70 with siRNA resulting in the restoration of NMT activity after 48 h further supports our observation that induction of NIP 71 beginning 72 h attenuates NMT activity. Regulation of NMT activity by hsc70 during U937 differentiation process assigns a novel function to it.

NMT is involved in the membrane anchoring and/or trafficking of various proteins including c-Src. Therefore, we followed the localization of c-Src during the differentiation of U937 cells. c-Src translocated into the Golgi apparatus during the differentiation process. To our knowledge, this is the first report that c-Src is predominantly Golgi associated, although a few previous reports have noted some limited association of c-Src with the Golgi apparatus. In addition, cells deficient in Src, Fyn, and Lyn have abnormal Golgi structure (46, 47). Molecular chaperone hsc70 plays a significant role in stabilizing the folding of the newly synthesized polypeptides in the endoplasmic reticulum (48, 49). Translocation of hsc70 during differentiation process along with c-Src may be due to the interaction of both the proteins. Thus, given hsc70’s known chaperone activity, it is likely that the trafficking of c-Src from cytoplasm to Golgi apparatus is aided by hsc70. Because heat shock proteins are also involved in intracellular protein transportation (50), interaction and colocalization of hsc70 and Src strongly suggests that Src is targeted to Golgi apparatus by hsc70. We also demonstrated that c-Src is localized in Golgi apparatus in BMDM indicating that c-Src resides in Golgi in differentiated macrophages. These results suggest that NMT1 may be essential for the monocytic/macrophage differentiation.

This study is the first of its kind and will lead to further insight in to the molecular basis of differentiation process. Macrophages belonging to the monocyte/macrophage lineage have long been recognized as one of the important components of the immune system involved in the host defense against malignancy (51–53). A better understanding of the macrophage development and its activation will help in understanding the molecular basis of immune response under cancerous or diseased state.

Disclosures

The authors have no financial conflict of interest.

References

analysis, and transcriptomics is a powerful tool for the identification of biomarkers for macrophage maturation in the U937 cell line. *Proteomics* 4: 1014–1028.


