Inhibition of JAK2/STAT3 signalling induces colorectal cancer cell apoptosis via mitochondrial pathway

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Abstract

Abnormalities in the JAK2/STAT3 pathway are involved in the pathogenesis of colorectal cancer (CRC), including apoptosis. However, the exact mechanism by which dysregulated JAK2/STAT3 signalling contributes to the apoptosis has not been clarified. To investigate the role of both JAK2 and STAT3 in the mechanism underlying CRC apoptosis, we inhibited JAK2 with AG490 and depleted STAT3 with a small interfering RNA. Our data showed that inhibition of JAK2/STAT3 signalling induced CRC cellular apoptosis via modulating the Bcl-2 gene family, promoting the loss of mitochondrial transmembrane potential (ΔΨm) and the increase of reactive oxygen species. In addition, our results demonstrated that the translocation of cytochrome c (Cyt c), caspase activation and cleavage of poly (ADP-ribose) polymerase (PARP) were present in apoptotic CRC cells after down-regulation of JAK2/STAT3 signalling. Moreover, inhibition of JAK2/STAT3 signalling suppressed CRC xenograft tumour growth. We found that JAK2/STAT3 target genes were decreased; meanwhile caspase cascade was activated in xenograft tumours. Our findings illustrated the biological significance of JAK2/STAT3 signalling in CRC apoptosis, and provided novel evidence that inhibition of JAK2/STAT3 induced apoptosis via the mitochondrial apoptotic pathway. Therefore, JAK2/STAT3 signalling may be a potential target for therapy of CRC.

Keywords: JAK2/STAT3 • apoptosis • mitochondrial apoptotic pathway • colorectal cancer

Introduction

Colorectal cancer (CRC) is a commonly diagnosed malignancy and a major cause of cancer-related death worldwide [1–3]. Despite growing understanding of oncogenesis and successful identification of proto-oncogenes and tumour suppressor genes involved in the tumourigenesis of CRC, the biological and molecular mechanisms in CRC are poorly understood. Recently, accumulated evidence has shown that abnormalities in the Janus kinase 2/ signal transducer and activator of transcription3 (JAK2/STAT3) signalling pathway are associated with the oncogenesis of several cancers. For example, some studies showed that sustained activation of the JAK2 is a causal event [4, 5]. Moreover, constitutive activation of STAT3 correlates with cell proliferation in non-small cell lung cancer [6] and pancreatic cancer [7]. Conversely, several studies have demonstrated the proof of principle that modulating JAK2/STAT3 signalling arrests the growth of primary human cancer cells [8] and promotes anti-tumour immunity in experimental cancer models [9]. Additionally, excellent progress has been made in exploiting STAT3 as a drug target in several forms of cancer [10]. Recently, some studies have also found that inhibition of JAK2,3/STAT3 signalling induces apoptosis in CRC cells [11, 12].

Deregulation of apoptosis is an important aspect of cancer pathogenesis and has been widely recognized as a hallmark of most types of cancer [13]. There are two key molecular signalling pathways that lead to apoptotic cell death. The first is the mitochondrial-mediated pathway [14], which is activated from...
inside the cell by members of Bcl-2 family and downstream mitochondrial signals. The second is the death receptor-mediated pathway [15], which is activated from outside the cell by proapoptotic ligands binding to specialized cell surface death receptors. Both apoptosis pathways converge at the level of activation of effector caspases activation, a family of cysteine proteases that are present in most cells as inactivezymogens, and become activated following a death signal. Activated caspases cleave cellular substrates and carry out numerous proteolytic events that mediate the apoptotic cell death program [16, 17]. Our previous study indicated that JAK2/STAT3 signalling is involved in CRC cell growth and survival through regulating expression of genes, such as Bcl-2 [11]. However, no essential studies on the exact molecular mechanisms of the apoptosis induced by inhibition of JAK2/STAT3 in CRC are available. Since Bcl-2 can inhibit cytochrome c (Cyt c) release and prevent opening of the megachannel by acting on the mitochondria [18], we speculated that the mitochondrial-mediated pathway may be involved in JAK2/STAT3 signalling induced apoptosis.

In this study, we used a fluorescent probe JC-1 or DCF fluorescence to detected Δψm or reactive oxygen species (ROS), respectively. The changes in expression of several proteins that are directly related to the mitochondrial-mediated pathway, such as Cyt c, caspases and Poly (ADP-ribose) polymerase (PARP), were also evaluated. In addition, we tested the hypothesis that JAK2/STAT3 signalling pathway could serve as a therapeutic targets in CRC xenograft models. Our findings illustrate the biological significance of JAK2/STAT3 signalling in CRC apoptosis, and provide novel evidence that inhibition of JAK2/STAT3 induces apoptosis via the mitochondrial apoptotic pathway.

Materials and methods

Cell culture, treatment with pharmacologic agents and transient transfection of STAT3 siRNA

Two human CRC cell lines SW1116 and HT29 were cultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) and McCoy’s 5A medium (Gibco), respectively, both supplemented with 10% foetal bovine serum at 37°C in a humidified atmosphere with 5% CO2 supplemented. AG490 (Sigma-Aldrich, St. Louis, MO, USA), a pharmacological JAK2 inhibitor, was dissolved in ethanol and used as the control. Commercial STAT3 siRNA (100 nM) used to target CRC cells was transfected with the DharmaFECT 1 transfection reagent (Thermo Scientific Dharmacon Inc., Lafayette, CO, USA). Cells transfected with non-specific siRNA (Thermo Scientific Dharmacon Inc.) were used as negative controls (NC).

Western blots

Western blot analysis to determine the levels of various proteins was performed using standard techniques as described previously [4]. For loading control, the membrane was probed with a monoclonal antibody for α-tubulin. Antibodies used in this study were purchased from Cell Signaling Technology, Danvers, MA, USA.

Detection of apoptosis

Mid-stage and late-stage apoptosis was determined by flow cytometry analysis, using annexin-V FITC/propidium iodide double staining assay in accordance with the manufacturer’s protocol (Becton Dickinson Biosciences, Bedford, MA, USA).

Detection of mitochondrial membrane potential

To clarify whether the observed apoptosis was related to the changes of mitochondrial membrane permeability, we used the fluorescent probe JC-1 (Invitrogen, Carlsbad, CA, USA) to measure the Δψm of CRC cells according to the manufacturer’s directions. Cells cultured in six-well plates after treatment with AG490 or transient transfection with siRNA for 48 hrs or 72 hrs, respectively, were incubated with JC-1 staining solution (10 μg/ml) at 37°C for 10 min. The fluorescence intensities of both mitochondrial JC-1 monomers (λex, 495 nm; λem, 530 nm) and aggregates (λex, 545 nm; λem, 590 nm) were detected using the LSM510 confocal fluorescent microscope (ZEISS, Germany) and analysed with Image J software. The Δψm of CRC cells in each treatment group was calculated as the ratio of the intensity of green (i.e. monomers) to that of red (i.e. aggregates) fluorescence [19].

Determination of intracellular ROS generation

To further evaluate changes in mitochondria, we assessed the intracellular concentration of ROS by using the non-fluorescent probe 2’,7’-dichlorofluorescein diacetate (DCFH-DA). The CRC cells were incubated with DCFH-DA at 37°C for 20 min. in dark conditions after treatment with AG490 for 48 hrs or being transient transfection with STAT3 siRNA for 72 hrs. Signals were recorded by using a fluorescence microscope (Olympus IMT-2, Japan). The intracellular ROS concentration was quantified by the measurement of the fluorescence intensity with Image J software.

Cyt c translocation

As transmutation of mitochondrial membrane permeability can induce Cyt c translocation, we monitored the shift in Cyt c from the mitochondria to the cytosol using the Cell Mitochondria Isolation Kit (Beyotime Institute of Biotechnology, China). Samples of cytosol and mitochondria were dissolved in lyses buffer and probed with an antibody against Cyt c (Cell Signaling Technology).

In vivo experiments

The CRC xenograft models were used to test the hypothesis that JAK2/STAT3 signalling could serve as therapeutic targets. SW1116 cells (1.0×10⁶) were injected subcutaneously into the dorsal right flank of 4-week-old male BALB/c nude mice (Experimental Animal Centre of SIBS) to establish the CRC xenograft model. After the tumour diameter reached 5 mm, mice were randomly allocated (6 mice/group) and were treated by injection with AG490 intraperitoneally at 10 mg/kg (low dose group) or 15 mg/kg (high dose group) for 10 days and by way of multipoint intratumoral injection (10 μg/30 μl per tumour) of siRNA complexed with transfection reagent in vivo jetPEI (Polyplus-transfection Inc., NY, USA) [20] every other day for 11 days. Tumour volume (mm³) was estimated by the
The formula for calculating tumour volume is: \[ \text{tumour volume (mm}^3) = \text{shorter diameter}^2 \times \text{longer diameter}/2. \]

The tumour volumes data are presented as means ± SD. In addition, Western blotting was performed to examine JAK2/STAT3 signalling activation and the activation of the caspase cascade in xenograft tumour tissues. All experimental procedures were approved by the Institutional Animal Care and Use Committee.

**Statistical analysis**

Results are shown as means ± SD. Variance (ANOVA) was used to determine statistical significance among all groups. \( P < 0.05 \) was considered statistically significant. All analyses were performed using SPSS for Windows 11.0.1 software.

**Results**

**Inhibition of JAK2/STAT3 signalling induces CRC cell apoptosis through regulation of Bcl-2 family genes**

Western blot analysis showed a time-dependent decrease in the expression of JAK2 and pJAK2 after treatment with AG490 (Fig. 1A). Meanwhile, AG490 exposure also decreased pJAK1 levels in both CRC cell lines (Fig. S1A). Nevertheless, no significant changes in the JAK3 and pJAK3 levels were detected by AG490 treatment (Fig. S1A). In addition, AG490 decreased the pSTAT3 levels in a time-dependent manner in SW1116 and HT29 cells. However, no detectable changes in the STAT3 level were seen in AG490-treated cells. Therefore, we used a siRNA to selectively reduce the expression of STAT3. Western blot analysis revealed that both STAT3 and pSTAT3 were depleted in CRC cells (Fig. 1B). Moreover, siRNA-mediated suppression of STAT3 did not affect the expression of STAT5 or pstat5 (Fig. S1B).

To explore the effects of JAK2/STAT3 signalling on CRC cell apoptosis we performed flow cytometry analysis (Fig. 2A). Our studies demonstrated that there was a 17.6-fold increase in apoptotic SW1116 cells and a 33.5-fold increase in apoptotic HT29 cells after 48 hrs of treatment with 100 \( \mu \)M AG490. STAT3 siRNA treatment also induced similar effects at 72 hrs after transfection when apoptosis of SW1116 and HT29 cells increased by 4.43- and 7.14-fold, respectively, higher than that in the control cells transfected for the same period with nonspecific siRNA. We further distinguished apoptosis from necrosis in these cells by flow cytometry [21]. The proportions of necrotic SW1116 and HT29 cells were 1.3% and 0.7%, respectively, after transient transfection with control siRNA. Meanwhile, the proportions of necrotic...
SW1116 and HT29 cells were 3.4% and 1.8% respectively, after transient transfection with STAT3 siRNA. These results showed that there were no significant differences in the levels of necrosis in STAT3 siRNA-treated cells compared with the control in both CRC cell lines (Fig. S3). Together, these data indicated that JAK2/STAT3 signalling is involved in the apoptotic process of CRC cells.

To ascertain the molecular mechanism involved in the above process, we examined the expression levels of apoptosis related proteins known to be downstream targets of the JAK2/STAT3 pathway. AG490 induced down-regulation of Bcl-2 simultaneously with the up-regulation of Bax in a time-dependent manner in both SW1116 and HT29 cells (Fig. 2B). In addition, similar effects were detected by transfection with siRNA for 72 hrs to deplete STAT3 (Fig. 2C). Therefore, inhibition of JAK2/STAT3 signalling induces apoptosis by altering the balance of the protein levels of these pro- and anti-apoptotic molecules in CRC cells.

Disruption of JAK2/STAT3 signalling promotes the loss of mitochondrial transmembrane potential ($\Delta\psi_m$) and increase of ROS

Since many apoptosis signals originating from the mitochondria are regulated through the Bcl-2 family [22], we hypothesized that the modulation of Bcl-2 and Bax would result in the changes of the mitochondrial transmembrane permeability. To further characterize the molecular mechanisms of JAK2/STAT3 signalling mediated apoptosis, we measured $\Delta\psi_m$ with in situ JC-1 staining after AG490 or STAT3 siRNA treatment. Control cells treated with the equivalent ethanol volume and then stained with JC-1 emitted mitochondrial orange-red fluorescence with a small degree of green fluorescence indicating hyperpolarized mitochondria. However, aggregation of JC-1 within normal mitochondria was replaced by diffuse green monomer fluorescence after treatment with AG490 (Fig. 3A). Additionally, the same phenomenon was
detected after transfection with STAT3 siRNA (Fig. 3A). Therefore, the data suggested the occurrence of depolarization of mitochondria and reduction of $\Delta \psi \text{m}$ in both SW1116 and HT29 cell lines by disruption of JAK2/STAT3 signalling.

On the other hand, ROS can be scavenged by the members of the Bcl-2 family, and up-regulation of ROS also leads to a reduction of $\Delta \psi \text{m}$ [23]. Therefore, we further determined whether the changes in the Bcl-2 family could induce the alteration in ROS and whether the reduction of $\Delta \psi \text{m}$ was partly induced by the up-regulation of ROS. We found significant increases in the intracellular concentration of ROS in both SW1116 cells and HT29 cells after AG490 or STAT3 siRNA treatment (Fig. 3C). Thus, the results demonstrated that the apoptosis induced by inhibition of JAK2/STAT3 signalling may be associated with the mitochondrial pathway.

**Inhibition of JAK2/STAT3 signalling induces the translocation of mitochondrial Cyt c to the cytosol**

The alteration of $\Delta \psi \text{m}$ causes mitochondria to become permeable by opening the mitochondrial permeability transition pores, which is a pathway for the release of the apoptogenic protein Cyt c from mitochondria into the cytoplasm [24]. Therefore, we detected the Cyt c release after disruption of JAK2/STAT3 signalling. Western blot analysis showed a time-dependent increase in the expression of Cyt c in cytosol after treatment with 100 $\mu$M AG490 (Fig. 4A). Cyt c was almost undetectable in cytosol before treatment with AG490 in SW1116 cells and the overall pattern was similar in HT29 cells. Meanwhile, AG490 exposure also decreased in Cyt c...
levels in mitochondria of both CRC cell lines. Cyt c expression altered sharply after 48 hrs of exposure to AG490 in both cytosol and mitochondria, suggesting that JAK2 may play an important role in regulation of Cyt c translocation. To further evaluate the role of STAT3, we down-regulated its expression by siRNA. Our data suggested that STAT3 participated in Cyt c translocation from mitochondria to cytosol at 72 hrs post-transfection (Fig. 4B).

Inhibition of JAK2/STAT3 signalling induces the caspases cascade and PARP cleavage

Since the translocation of Cyt c can activate the caspase cascade [25], we further explored whether caspases were involved after inhibition of JAK2/STAT3 signalling. As illustrated in Figure 5A, pretreatment of CRC cells with AG490 decreased the expressions of pro-caspase9 and pro-caspase3, meanwhile increased the expression of cleaved-caspase9 and cleaved-caspase3. We also found that the most significant alteration emerged at 72 hrs after exposure to AG490. STAT3 siRNA induced similar effects. At 72 hrs after transient transfection with 100 nM of STAT3 siRNA, the expressions of pro-caspases3,9 of SW1116 and HT29 cells decreased and cleaved-caspases3,9 increased when compared with the equivalent transfection with nonspecific siRNA (Fig. 5B). However, the active caspase-8 involved in the death receptor pathway was not significantly increased, after transient transfection with STAT3 siRNA for 72 hrs in both CRC cell lines (Fig. S2). Our results suggested that the caspase cascade involved in the mitochondrial pathway is activated after inhibition of JAK2/STAT3 signalling.

To further explore the effects of JAK/STAT signalling on the caspase cascade, we detected PARP, a substrate for caspase-3, which is known to be a key substrate associated with apoptosis at the nuclear level [26]. As seen in our results, PARP was cleaved after 24 hrs of AG490 treatment in both CRC cell lines. STAT3 siRNA displayed the same phenomenon compared with NC. Moreover, cleaved-PARP was almost undetectable after transfection with nonspecific siRNA.

Inhibition of JAK2/STAT3 signalling suppresses CRC xenograft tumour growth

To determine whether components of JAK2/STAT3 could serve as therapeutic targets, we established a tumour model in nude mice bearing SW1116 xenografts. After treatment with different concentrations of AG490, we found that the tumour volume of the high dose group (15 mg/kg) was smaller than that of the low dose group (10 mg/kg) without obviously toxicity (P < 0.05). Moreover, transient transfection of STAT3 siRNA also led to the slower growth of SW1116 xenografts when compared with the mock or NC animal group (Fig. 6A).

Furthermore, we examined the protein levels of JAK2, pJAK2, STAT3 and pSTAT3 of CRC xenografts by Western blot. As shown in Fig. 6B, the JAK2/STAT3 signalling target proteins were all
decreased when compared with the mock group. Meanwhile, we investigated the level of activation of the caspase cascade after AG490 or STAT3 siRNA treatment. As shown in Figure 6B, changes of the JAK2/STAT3 pathway dramatically induced activation of the caspase cascade inside the tumours compared with the mock or NC group. Taken together, these data indicated that targeting JAK2/STAT3 could exert anti-tumour effects in vivo on CRC and may serve as a therapeutic target.

**Discussion**

Abnormalities of the JAK/STAT pathway are involved in the pathogenesis of several cancers [6] including apoptosis. However, the molecular mechanism by which disordered JAK2/STAT3 signalling contributes to apoptosis has not been clarified. Therefore, understanding the mechanisms of apoptosis is crucial to comprehending the role of the JAK2/STAT3 pathway in cancer therapies. Our previous work found that inhibition of JAK2/STAT3 signalling induced CRC cells apoptosis. However, the exact mechanisms involved are still incompletely understood. In our study, we used AG490 to inhibit JAK2, pJAK2 and pSTAT3 (Fig. 1). Although AG490 has been widely used for inhibiting JAK2, it has unspecific inhibit effects. To further demonstrate the exactly apoptosis pathway involved in the CRC cells after inhibition of STAT3, we used STAT3 siRNA to knock down STAT3.

There are many areas of uncertainty related to mechanisms of apoptosis, including by overexpressing pro-apoptotic proteins and inducing the signalling cascade at various levels. Our previous study also found that the expression of Bcl-2 proteins that are known to be downstream targets of the STATs pathway is decreased after suppression of JAK2/STAT3 signalling. Therefore, in this study we detected the members of the Bcl-2 family, the anti-apoptosis protein Bcl-2 and the pro-apoptosis protein Bax. The down-regulation of Bcl-2 and up-regulation of Bax were observed in a time-dependent manner in CRC cells after alteration of JAK2/STAT3 signalling (Fig. 2).
The permeabilization of the mitochondrial outer membrane is tightly controlled by Bcl-2 [21, 27], and activation of Bax can trigger a sequence of events that leads to alterations in mitochondrial permeability transition [28]. Therefore, we turned our attention to and found that reduction of Δψm emerged in both CRC cells after inhibition of JAK2/STAT3 signalling as indicated by an increase in the green/red ratio (Fig. 3A). The results indicated that the changes of mitochondrial outer membrane permeabilization constitute one of the major checkpoints of JAK2/STAT3 signalling as indicated by an increase in the green/red ratio (Fig. 3A). The results indicated that the changes of mitochondrial outer membrane permeabilization constitute one of the major checkpoints of JAK2/STAT3 signalling as indicated by an increase in the green/red ratio (Fig. 3A). 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observed activation of the caspase-9 after treatment with AG490 in a time-dependent manner and after transient transfection of STAT3 siRNA. The activation of caspase-9 subsequently stimulated downstream caspases-3, which were activated through the mitochondrial signalling pathway. Caspase-3 cleaves several substrates, such as PARP, resulting in chromosomal DNA fragmentation and cellular apoptosis, which were also showed in our study. All of these results demonstrated that inhibition of JAK2/STAT3 signalling could down-regulated Δψm via modulating the expression of Bcl-2 family genes and oxidative stress. Moreover, the down-regulation of Δψm causes Cyt c to be translocated from the mitochondria to the cytoplasm to activate downstream caspase cascade and PARP, which ultimately leads to apoptosis in CRC cells (summarized in Fig. 7).

To test the hypothesis that JAK2/STAT3 signalling could serve as a therapeutic target, we constructed a nude mouse CRC cell xenograft model. SW1116 cells are widely used in tumour xenograft model [33, 34] and were injected subcutaneously into the dorsal right flank of nude mice. AG490 and STAT3 siRNA were shown to cause marked tumour regression without apparent toxicities in these mice. These results could be explained by either the down-regulation of target genes or by the activation of the caspase cascade after inhibition of the JAK2/STAT3 signalling. Our results demonstrate that JAK2/STAT3 signalling could be a potential therapeutic target in CRC. Although tumour subcutaneous xenograft model is useful for enhancing our understanding of cancer development and treatment, there are several disadvantages and challenges of using the mouse xenograft model to monitor and/or predict therapeutic responses in cancer. Therefore, primary human tumours and further studies are needed to validate this hypothesis.

Since mitochondrial pathway is not the only apoptosis pathway involved in cancer cell death, we also detected whether death receptor pathway participated in AG490 and STAT3 induced CRC cells apoptosis. Death inducing signalling complex induced the activation of caspase-8, which in turn precipitated the activation of downstream effector caspases in the death receptors pathway. Therefore, we detected the activation of caspase-8 by ELISA. Our result showed that there were no significant differences after transient transfection of STAT3 siRNA in both CRC cell lines (Fig. S2). Therefore, STAT3 siRNA induced CRC cells apoptosis mainly via the mitochondrial pathway.

Both apoptosis and necrosis are two important mechanisms of cell death. We further considered whether inhibition of JAK2/STAT3 signalling could induce CRC cell necrosis. In order to do so, it was necessary to quantify and distinguish between apoptotic and necrotic cells using a flow cytometric method. Although it seemed that there were more necrotic cells in the STAT3 siRNA treatment group than the NC, the differences were not significant in either CRC cell line (Fig. S3). Based on these results, STAT3 siRNA induced cell death mainly through apoptosis.

In conclusion, this study is the first to have examined in detail the molecular mechanism of JAK2/STAT3 signalling in CRC apoptosis. Our present findings suggest that inhibition of JAK2/STAT3 signalling induces CRC apoptosis via the mitochondrial pathway. Thus, our data provide further support for targeting the JAK2/STAT3 signalling in the potential treatment of human CRC.

**Ethics statement**

Our study was approved by the Animal Care and Use Committee of the Shanghai Jiao-Tong University School of Medicine Renji Hospital, Shanghai, China. All animal procedures were performed according to the guidelines developed by the China Council on Animal Care and the protocol approved by the Shanghai Jiao-Tong University School of Medicine, Renji Hospital, Shanghai, China.
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Conflict of interest

The authors confirm that there are no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Fig. S1 Effect of AG490 and STAT3 siRNA on JAK/STAT pathway genes. (A) Western blot analysis revealed that AG490 induced a decrease in pJAK1 levels at 24 hrs in CRC cells. In the same experiment, JAK1, JAK3 and pJAK3 also identified although these decreases were not statistically significant. (B) At 72 hrs post-transfection, Western blot analysis showed that STAT3 siRNA did not affect the expressions of STAT5 and pSTAT5. The data shown are representative of three separate experiments. α-tubulin was used for the loading control. Quantification of the target protein bands relative to tubulin is shown in the right panel.

Fig. S2 Inhibition of STAT3-induced CRC cells apoptosis may not be associated with the death receptor pathway. Activated caspase-8 showed no significant difference between control siRNA group and STAT3 siRNA group as detected by ELISA in both CRC cell lines after transient transfection for 72 hrs.

Fig. S3 Suppression of STAT3 does not induce CRC cell necrosis. CRC cells were treated with STAT3 siRNA for 72 hrs and cell apoptosis and necrosis were detected by flow cytometric analysis. The lower left quadrant, the upper and lower right quadrant and the upper left quadrant represent viable cells, apoptotic cells and necrotic cells, respectively. Although the number of the necrotic cells in the STAT3 siRNA treatment group seemed to be greater than that of negative control, these differences were not significant in either CRC cell line.

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