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The extracellular matrix, p53 and estrogen compete to regulate cell-surface Fas/Apo-1 suicide receptor expression in proliferating embryonic cerebral cortical precursors, and reciprocally, Fas-ligand modifies estrogen control of cell-cycle proteins

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Abstract

Background: Apoptosis is important for normal cerebral cortical development. We previously showed that the Fas suicide receptor was expressed within the developing cerebral cortex, and that *in vitro* Fas activation resulted in caspase-dependent death. Alterations in cell-surface Fas expression may significantly influence cortical development. Therefore, in the following studies, we sought to identify developmentally relevant cell biological processes that regulate cell-surface Fas expression and reciprocal consequences of Fas receptor activation.

Results: Flow-cytometric analyses identified two distinct neural sub-populations that expressed Fas on their cell surface at high (Fas^{Hi}) or moderate (Fas^{Mod}) levels. The anti-apoptotic protein FLIP further delineated a subset of Fas-expressing cells with potential apoptosis-resistance. Fas^{Mod} precursors were mainly in G0, while Fas^{Hi} precursors were largely apoptotic. However, birth-date analysis indicated that neuroblasts express the highest levels of cell-surface Fas at the end of S-phase, or after their final round of mitosis, suggesting that Fas expression is induced at cell cycle checkpoints or during interkinetic nuclear movements. Fas^{Hi} expression was associated with loss of cell-matrix adhesion and anoikis. Activation of the transcription factor p53 was associated with induction of Fas expression, while the gonadal hormone estrogen antagonistically suppressed cell-surface Fas expression. Estrogen also induced entry into S-phase and decreased the number of Fas-expressing neuroblasts that were apoptotic. Concurrent exposure to estrogen and to soluble Fas-ligand (sFasL) suppressed p21/waf-1 and PCNA. In contrast, estrogen and sFasL, individually and together, induced cyclin-A expression, suggesting activation of compensatory survival mechanisms.

Conclusions: Embryonic cortical neuronal precursors are intrinsically heterogeneous with respect to Fas suicide-sensitivity. Competing intrinsic (p53, cell cycle, FLIP expression), proximal (extra-cellular matrix) and extrinsic factors (gonadal hormones) collectively regulate Fas suicide-sensitivity either during neurogenesis, or possibly during neuronal migration, and may ultimately determine which neuroblasts successfully contribute neurons to the differentiating cortical plate.

Background

The developing cerebral cortex and other brain regions undergo substantial cell suicide during the period of neurogenesis and early differentiation [1-10], to generate a mature brain. Mechanisms that control the survival or death of neuroblasts and neurons in the developing cerebral cortex are likely to have profound effects on the organization of cognition, affect and sensori-motor integration in the adult. One generally accepted mechanism that is invoked to explain developmental apoptosis is the competition among neurons for limited supplies of trophic molecules within the environment (reviewed in [11]). According to this model, a neuron's inability to find growth factor support within its environment precedes the initiation of apoptosis. However, the presence of cell-surface suicide receptors, like the Fas/Apo [Apoptosis]-1/CD95 receptor, and their trans-membrane ligands (e.g., FasL) in the developing brain [9] suggests that neural cells may actively communicate apoptosis signals to each other. In addition to competing for a limited supply of trophic factors, developing neural progenitors and differentiating neurons may engage in an active killing process whereby 'killer cells' induce apoptosis in 'suicide-receptive' cells, to limit cell number in the brain. It is therefore important to understand the signaling mechanisms and circumstances that regulate cell-suicide receptor expression in the developing brain.

The Fas cell-suicide receptor plays an important role in limiting cell proliferation in the immune system by apoptosis [12]. Recent evidence suggests that Fas is also an important regulator of cell death in the brain. Fas is expressed by the developing cerebral cortex during the peak period of apoptosis [7,9,13], and by other differentiating neural cells [14-18]. Fas is also re-expressed in neurological disease conditions including ischemia, multiple sclerosis, Alzheimer's disease, and in neural tumors [15,19-27]. We previously reported that Fas activation leads to unscheduled DNA synthesis, the activation of NF-κB, and caspase-dependent cell death in embryonic cortical neuroblasts [9]. Neuronal cultures obtained from developing *gld* (Fas-deficient) mice are less sensitive to apoptosis signals than wild-type controls [17]. Finally, haplo-insufficiency of the tyrosine phosphatase *Pten*/MMAC1 results in the inactivation of Fas [28], resistance to apoptosis in neural progenitors [29], and consequently, an increased incidence of tumors of neural origin [30]. These data indicate that the Fas/Apo-1 suicide system may determine cell number in the brain, both during development, and following injury in the adult. However, we know very little about the types of neural progenitors and neuronal cells that are particularly vulnerable to Fas-induced death. We also know little about the signaling mechanisms in differentiating neural progenitors that promote cell-surface expression of Fas, and consequently, sensitivity to apoptosis.

The transcription factor p53 is important for brain development [31]. In lung and prostate cancer cell lines, Fas activation is dependent on the presence of functional p53 [32]. P53 induces Fas gene transcription [33], the translocation of Fas from Golgi complex to cell surface [34] and activates Fas-mediated apoptosis in un-transformed vascular smooth muscle cells [34] and mammary epithelial tumors [35]. In the adult rat hippocampus, seizure leads to the co-localized expression of p53 and Fas in CA1 neurons [36]. However, it is likely that p53 acts in concert with a coterie of transcription factors to regulate the availability of Fas in the developing brain. For example, we found that estrogen promotes p53 phosphorylation [37], but uncouples p53 from its regulation of Bax, a mitochondrial-associated pro-apoptotic factor. Furthermore, estrogen receptors block the DNA binding activity of NF-kB [38], a transcription factor downstream of members of the TNFr/Fas family [9,39]. Thus estrogen, and consequently estrogen receptors, may be an important regulator of Fas expression in the developing cerebral cortex.

In the following experiments, we set out to identify cell biological processes that predict cell-surface expression of Fas, and to identify consequences of Fas activation. Our experiments focused predominantly on the pool of Fas receptors that localized to the cell surface, because this population constitutes the bioactive proportion of Fas receptors that contributes to apoptosis sensitivity. We hypothesized that, in embryonic cerebral cortex, cell-surface Fas expression would be associated with exit from cell cycle, and activation of p53. We also hypothesized that estrogen controls neurogenesis and neuron elimination, either by regulating the Fas receptor, or by regulating specific anti-apoptotic cell cycle elements in a Fas-dependent manner.

Our results indicate that embryonic cortical precursors are heterogeneous with respect to cell surface Fas expression and the co-expression of the anti-apoptotic protein FLIP (FLICE/caspase-8 interacting protein). Cell-surface Fas expression in embryonic cortical neurons is associated with recent exit from cell cycle, and with loss of contact with the extra-cellular matrix and anoikis. Fas expression is coincidentally increased following p53 activation. In contrast, estrogen decreases Fas expression, and following the activation of Fas by soluble Fas-ligand (sFasL), estrogen suppresses expression of p21/Waf-1/Cip-1 and the proliferating cell nuclear antigen (PCNA). Estrogen and sFasL separately and together induce expression of cyclin A.

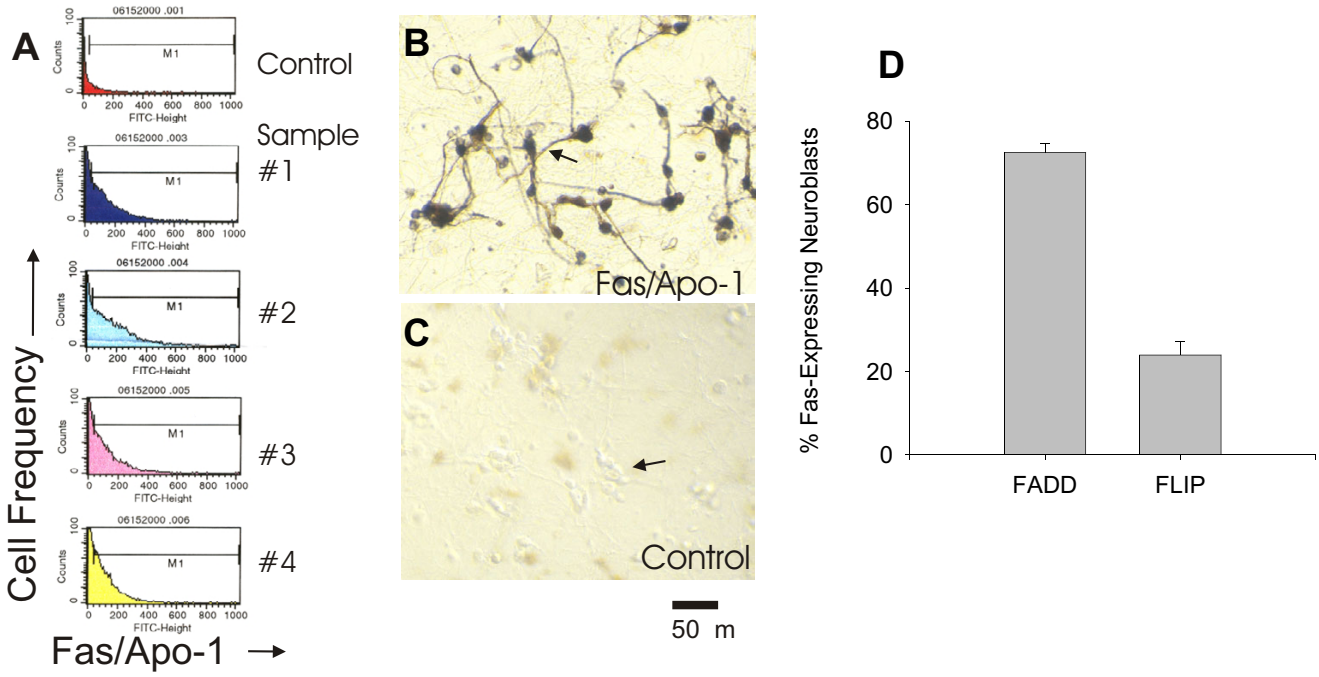

Figure 1
(A) Flow cytometric analyses of independent samples GD15 cortex show more cells expressing Fas immuno-fluorescence in samples #1–#4, compared to pre-immune serum control. Gate 'M' was set to exclude background fluorescence, or 98% of neuroblasts in the control sample (B & C) immunohistochemical analysis of cultured embryonic cortical neurons indicates Fas-immunoreactivity is localized to the soma and proximal processes (arrow). Immunohistochemical controls (arrow, C) show lack of staining in neurons. (D) Graph (Mean ± SEM) of the percentage of Fas-expressing precursors that also co-localize the pro-apoptotic DISC adapter protein FADD, or the anti-apoptotic inhibitor FLIP.

Results

Expression of Fas and related proteins in embryonic cerebral cortical precursors

In the initial experiment, we utilized flow cytometric analysis to examine the localization of Fas to the cell surface of freshly isolated embryonic rat (gestational day (GD)-15) cortical neural cells by immuno-labeling for Fas in the absence of detergent. At this developmental age, the neuroepithelium proliferates rapidly and is the dominant cortical structure, and therefore, neuroepithelial precursors are the most abundant component of this tissue. Furthermore, at this age, the neuroepithelium mainly consists of neuronal (as opposed to glial) precursors (for review, see [40]). Flow cytometric analysis of samples obtained from GD15 rat cortex indicated that 10.69 ± 3.37% (mean ± SEM) of cortical neuronal precursors expressed Fas on their cell surface *in vivo* on embryonic day 15 (data obtained from 6 independent samples of E15 cortex, Figure 1A). Once cortical precursors are cultured however, immunohistochemical analysis of detergent-permeabilized cultures (a measure of total Fas expression) indicates that a majority of these differentiating cells localize Fas-immunoreactivity within their cytoplasm and proximal processes (Figure 1B vs. control Figure 1C and [9]). In contrast, flow cytometric analyses of non-permeabilized cultured embryonic cortical-derived neuronal precursors (Figure 2) indicate that 23–28% of these cells expressed Fas on their cell surface *in vitro*, representing ~2-fold induction of cell surface Fas expression *in vitro*, as compared to *in vivo*. Collectively, these data indicate that only a fraction (~$1/4^{th}$) of all Fas-expressing cells actually localize Fas to the cell-surface, *in vitro*.

Co-localization of Fas with FADD and FLIP

The protein FADD (Fas-Associated Death Domain) is the major intracellular death-inducing signaling complex (DISC) adapter protein that couples Fas to intracellular caspase pathways [41-44], while FLIP is the major proximal intracellular protein that blocks Fas/Apo-1-mediated apoptosis [45]. Since FADD and FLIP are antagonistic elements of the DISC-complex and are important intracellular gates of Fas/Apo-1 activation, we examined the extent to which these functionally divergent proteins co-localized with Fas/Apo-1 in differentiating embryonic cortical-

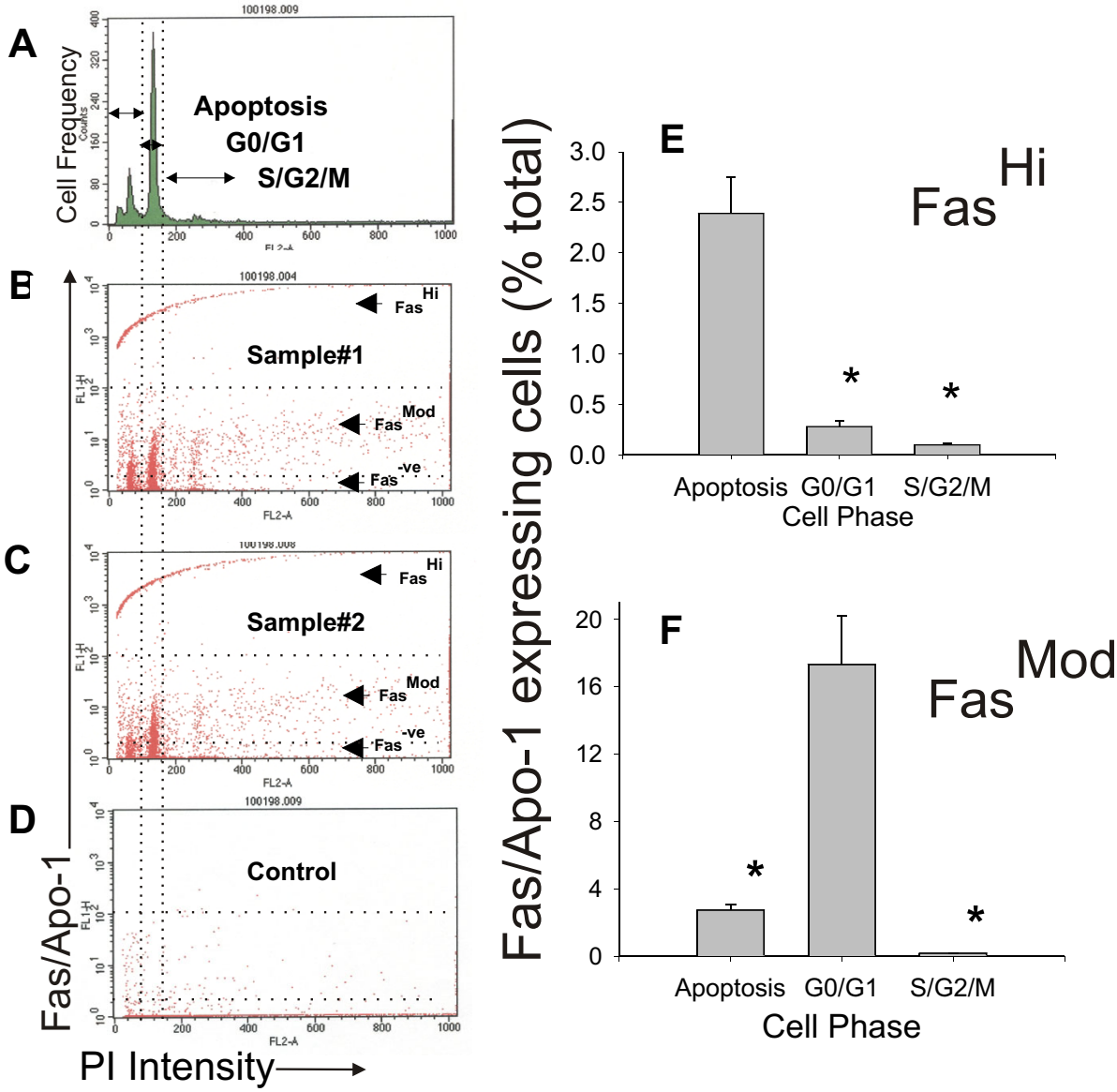

Figure 2
Identification of two unique populations of cell-surface Fas-expressing precursors (A-D). Flow cytometric analysis of the cell-cycle distribution of cultured embryonic cortical precursors (A), based on frequency histogram analysis of propidium iodide (PI) incorporation into DNA in 10,000 cells from each sample, indicates that a majority of cells precursors are in G0. Precursors with DNA content <G0 were identified as apoptotic, while precursors with >G0 DNA content were identified as being in S-G2-M. (B,C) Scatter-plots of two representative independent samples of embryonic cortical-derived neuronal precursors analyzed for combined cell-surface Fas immuno-fluorescence (y-axis) and PI incorporation (cell-cycle stage, x-axis). Based on background immuno-fluorescence patterns in the pre-immune serum controls (D), precursors were characterized as negative Fas-expressing (Fas^{-ve}, expressing $<2 \times 10^0$ fluorescence units [FUs]), moderate Fas-expressing (Fas^{Mod}, expressing $>2 \times 10^0$ and $<10^2$ FUs) and high cell surface Fas-expressing (Fas^{Hi}, $>10^2$ FUs). These categories remained consistent across experiments. (E & F) Graphical representation (Mean ± SEM) of the proportion of precursors expressing high (Fas^{Hi}, E) and moderate (Fas^{Mod}, F) levels of cell-surface Fas at different stages of cell cycle and apoptosis, expressed as a percentage of control. Asterisks indicate statistically significant differences in cell-stage-specific expression of cell surface Fas, $p < 0.05$.

derived neuroblasts. Two- parameter FACS analysis (Figure 1D) showed that, among adherent cultures, 72.5 ± 2.1% of Fas-expressing neuroblasts also co-localized FADD. In contrast, only 23.9 ± 3.2% Fas-expressing cells co-localized the anti-apoptotic protein FLIP suggesting that only a portion of Fas/Apo-1-expressing embryonic cortical-derived neuronal precursors may be protected from Fas-induced apoptosis.

Cell-cycle-related expression of Fas in embryonic cortical neuronal precursors

Using flow cytometric analyses, we identified three distinct populations of neural cells *in vitro*; based on differences in the intensity of cell-surface Fas expression (Figure 2). Approximately 3% of cortical cells expressed Fas on their cell surface at a high intensity (Fas^{Hi}), while 20% of cultured neural cells expressed moderate levels of Fas on their cell surface (Fas^{Mod}). In contrast, 77% of cultured cortical neural cells do not express Fas on their cell-surface above background levels (Fas^{-ve}). Cultured cortical cells were counterstained with propidium iodide (PI) to measure cellular DNA content and to stage Fas-expressing cells into apoptosis (defined as cells with less than G0 DNA content), cell cycle arrest (G0/G1) or cell cycle (G2/S/M) phases (Figure 2A,2B,2C,2D). Our results indicate that the largest proportion of neural cells in the Fas^{Hi} population were apoptotic (Figure 2E). Few Fas^{Hi} cells were in G0/G1 or S/G2/M stages of cell cycle. In contrast, within the Fas^{Mod} population, the greatest percentage of Fas-expressing cells was observed in G0/G1 (Figure 2F).

Functional determinants of Fas expression in cortical neuroblasts

The majority of Fas^{Hi} cells were apoptotic. We therefore examined the extent to which Fas expression was related to specific apoptosis-sensitive cell-biological processes including cell-matrix interactions, cell proliferation, and the activation of cell-cycle inhibitory proteins like p53.

Fas expression is dependent on cell-matrix interactions and 'anoikis'

The loss of adhesion to the extracellular matrix has been reported to induce apoptosis (a process referred to as anoikis), by activating caspases like caspase-2 [46] that are down-stream of suicide receptors like Fas/Apo-1. Such cell-matrix interactions appear to be contextually relevant to the developing brain as well, since deletion of the Matrix Metalloproteinase (MMP)-9 gene (consequently suppressing type-4 collagen degradation) leads to delayed migration of granule cells, and decreased apoptosis in the developing cerebellum [47]. Since post-mitotic neuroblasts migrate to their lamina-specific positions within the cortical plate using guidance cues that are dependent on the integrity of the extra-cellular matrix, we sought to determine if there was a relationship between cell-adhesion and Fas/Apo-1 expression. Non-adherent cells were harvested and analyzed separately from cells that were adherent to the culture dish. Among adherent cells (Figure 3A,3B), a majority of Fas-expressing neural cells belonged to the Fas^{Mod} group. In contrast, most Fas^{Hi} neural cells were non-adherent (Figure 3C,3D) and predominantly apoptotic.

Since primary cultured cells of the Fas^{Hi} group were predominantly non-adherent, we sought to determine if the induction of 'anoikis' could lead to an increase in Fas/Apo-1 expression. We therefore cultured embryonic day 15 cortical-derived neuroblasts on a collagen substrate (rat tail collagen, UBI) and treated adherent cultures with 50 U of Collagenase-A (MMP-1, microbial metalloendopeptidase, EC 3.4.24.3, Sigma) for 24 hours. Treatment of adherent neuronal precursors with collagenase-A led to a statistically significant, 2.5-fold increase in the number of cells that expressed Fas/Apo-1 on their cell surface (Figure 3E). Because of this relationship between 'anoikis' and Fas/Apo-1 expression, subsequent flow cytometric analyses focused on cells that were adherent to the extracellular matrix deposited onto the culture dish.

Cell surface Fas/Apo-1 expression is associated with cell cycle

We had previously observed that Fas activation was followed by a transient increase in the uptake of 5-Bromo-2'-deoxyuridine (BrdU, [9]) a marker of DNA replication. Though this increase in BrdU incorporation was not related to the induction of cell cycle, we hypothesized that the expression of Fas itself was related to cell proliferation and DNA synthesis. We therefore utilized three-parameter flow cytometric analyses to examine the relationship between cell-surface Fas expression and BrdU incorporation among neuronal precursors that were in cell cycle arrest (G0/G1), or actively in cell cycle (G2/S/M). Cells were first immuno-labeled for Fas, then detergent permeabilized and immuno-labeled for BrdU incorporation. Using propidium iodide (PI) fluorescence intensity as a classification parameter, cortical precursors were classified as belonging to one of three phases, G2/S/M, G0/G1, or apoptosis (sub-G0 DNA content). BrdU incorporation was significantly correlated with the cell-surface expression of Fas. In G0/G1 and S/G2/M conditions, correlation coefficients (r) ranged from 0.98 to 0.99, $p < 0.05$ (Figure 4A,4B). These data suggest that Fas/Apo-1 is most highly expressed in neuronal precursors that have recently completed their final cycle (for neuroblasts in G0/G1) or recently exited S-phase (for neuroblasts in S/G2/M). Furthermore, among cells in cell cycle (S/G2/M, Figure 4A), a discrete group of Fas^{Hi} precursors (arrows) also expressed the highest levels of BrdU incorporation. During cell cycle, the highest levels of BrdU incorporation are attained at the completion of chromosome replication

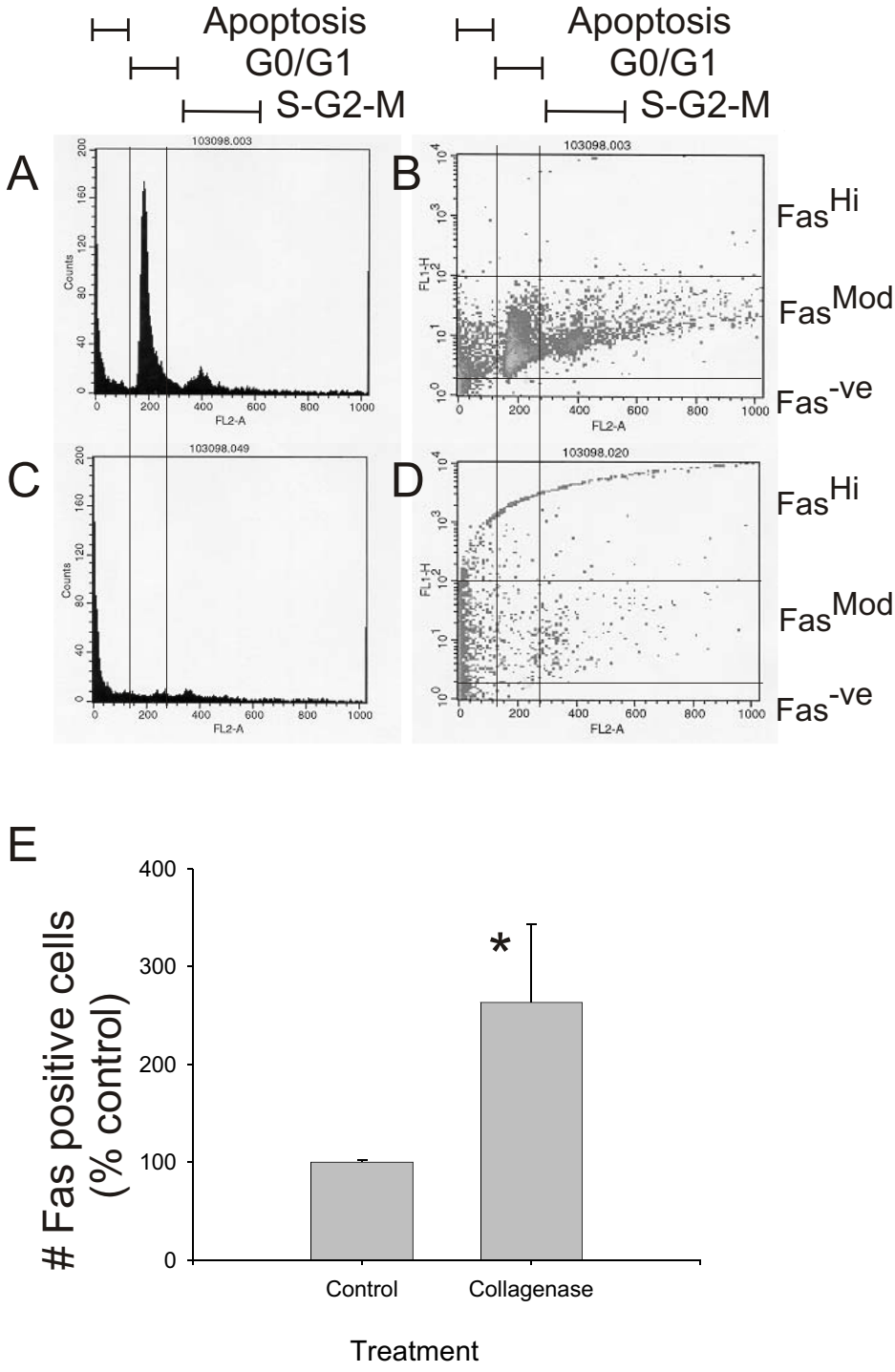

Figure 3
Relationship between Anoikis (apoptosis due to loss of cell adhesion) and cell surface Fas-expression. (A,C) Sample frequency histograms of PI incorporation (DNA content) in adherent (A) and non-adherent (C) precursors indicates that adherent precursors are mainly in G0, while non-adherent precursors are mainly apoptotic. (B, D) Scatter-plots of combined analysis of PI (x-axis) and cell-surface Fas immunofluorescence (y-axis) indicates that adherent precursors largely express moderate levels of cell-surface Fas (Fas^{Mod}, B), while non-adherent precursors also express high levels of Fas (Fas^{Hi}, D). (E) Graph (Mean + SEM) showing that collagenase-A treatment significantly increased the number of cells expressing cell-surface Fas, relative to controls. Asterisk indicates $p < 0.05$.

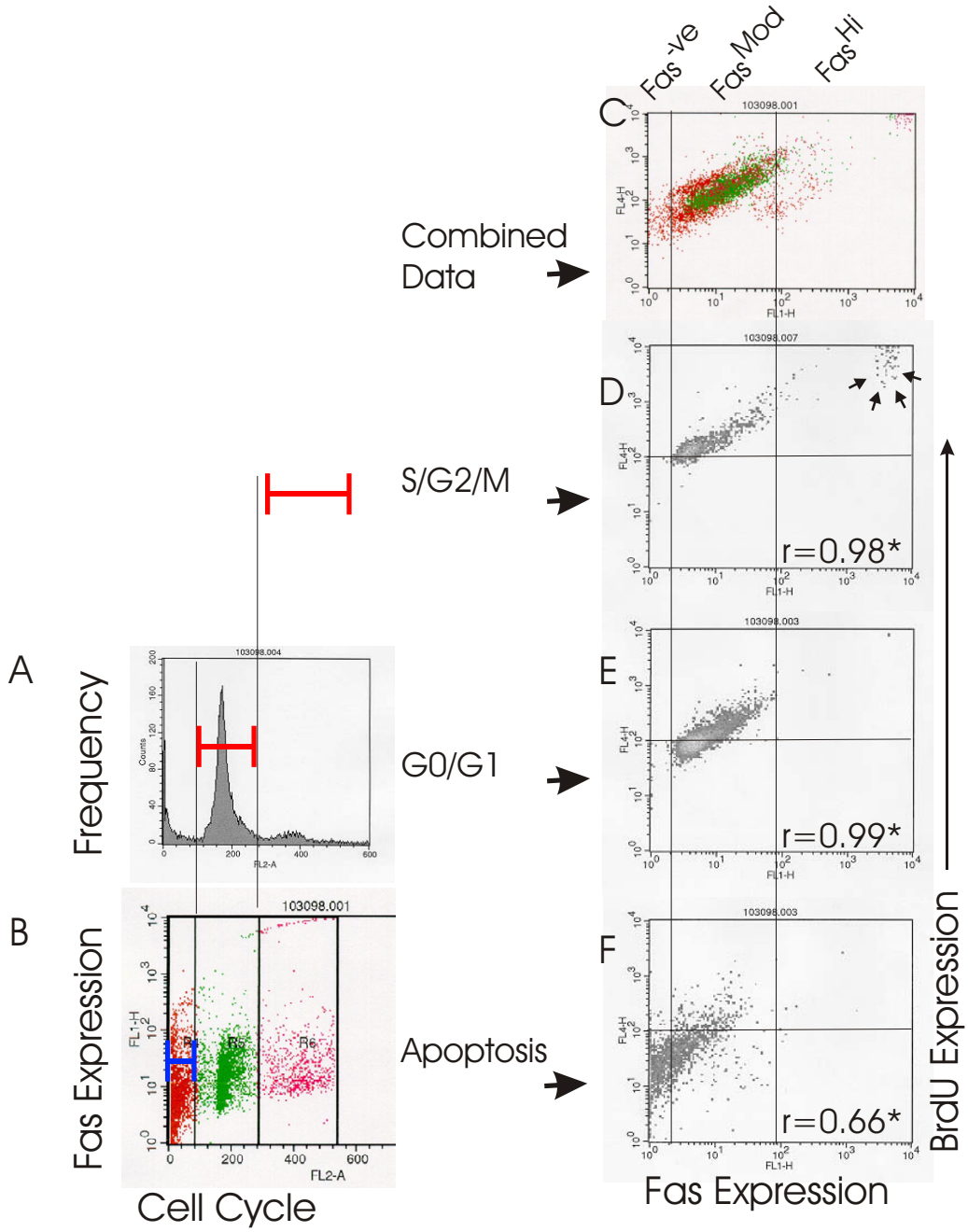

Figure 4
Relationship between cell-surface Fas expression and BrdU incorporation at different stages of cell cycle or apoptosis (PI incorporation) in adherent primary precursors. (A,B) Representative frequency histogram of PI incorporation (A) and scatter-plot of combined PI incorporation and Fas immunofluorescence (B) indicates the thresholds for the separate analysis of BrdU content in neuroblasts at S/G2/M (pink), G0 (green), or apoptosis (red). (C-F) Representative scatter-plots of the overall positive relationship between Fas expression and BrdU incorporation (C) and separated by cell-phase, S/G2/M (D), G0 (E) and apoptosis (F). Arrows in S/G2/M condition (D) indicate a group of cells that exhibited the highest levels of BrdU incorporation (suggesting recent completion of S-phase) and cell-surface Fas expression. Insets in figures D,E and F indicate Pearson's product moment correlations (obtained by averaging intensities across 6 independent samples). Asterisks indicate that the correlation was statistically significant with a 2-tailed test. These data indicate that the correlation between BrdU incorporation and cell-surface Fas expression is higher during cell cycle and G0 as compared to apoptosis.

(4N DNA content at the end of S-phase) and before the completion of mitosis. Therefore, G2 and early mitosis may represent periods during which some sub-populations of proliferating cortical neuroblasts are particularly vulnerable to receptor-mediated suicide signals. Our data therefore indicate that while Fas activation does not induce or suppress cell cycle [9], the highest expression of Fas is associated with progression past the S-phase of cell cycle or recent exit from cell cycle.

Fas expression in embryonic cortical-derived precursors is associated with p53 activation

The transcription factor p53 induces Fas gene transcription [33], the translocation of Fas from Golgi complex to cell surface [34], and activates Fas-mediated apoptosis in un-transformed vascular smooth muscle cells [34] and mammary epithelial tumors [35]. We had previously shown that p53 activation in cortical progenitor cells was associated with the induction of the mitochondrial death-associated protein Bax [37]. We therefore examined the extent to which p53 status predicted the expression of Fas/Apo-1 on the cell surface of cortical neuronal precursors. We used phosphorylation of p53 at serine 392 as a marker for p53 activation [37]. Cells were first immuno-labeled for Fas, then detergent-permeabilized and immuno-labeled for *p*(phospho)p53. The intensity of *p*p53 expression was statistically significantly correlated ($p < 0.05$, $r = 0.73$, Figure 5A) with the intensity of cell surface Fas expression in primary cortical neuronal precursors. Thus, cells that exhibit high levels of p53 phosphorylation also express high cell-surface levels of Fas.

As a follow-up experiment, to determine whether p53 activation is required for the expression of Fas/Apo-1, we utilized a rodent cerebral cortical cell line (CHB50), conditionally immortalized with a temperature sensitive mutation of the SV40 large T antigen (*ts*TA), that we had previously developed and characterized [37]. In the +*ts*TA condition (incubation at 33°C), neuroblasts proliferate indefinitely and p53 is inactive as indicated by the absence of phosphorylated p53 (-*p*p53). However, cessation of large T antigen expression (-*ts*TA, 39°C) is accompanied by p53 phosphorylation (+*p*p53) and the induction of p53-dependent proteins like Bax [37]. CHB50 neuroblasts were therefore cultured under +*ts*TA and -*ts*TA conditions. Western immunoblot analysis indicated that CHB50 neuroblasts expressed very low-to-undetectable levels of Fas in the -*p*p53 (+*ts*TA) condition. However, following the activation of p53, [+*p*p53 (-*ts*TA) condition], there was a statistically significant 12-fold increase in the expression of Fas, 48 hours following activation of p53 (Figure 5B,5C).

Estrogen regulation of Fas expression is cell-cycle dependent

We had previously observed that estrogen prevented apoptosis following p53 activation and decreased the expression of the mitochondrial-associated pro-apoptotic protein Bax in differentiating cerebral cortical neuroblasts [37]. Furthermore, in the context of p53 activation, estrogen led to an increase in neuroblast proliferation. We therefore examined the extent to which cell-surface Fas expression was estrogen-dependent.

Flow cytometric analysis of non-synchronized GD15 cortical neuronal precursors (Figure 6A) indicated that 24 hours exposure to estradiol-17β (E2) at 2 nM led to a statistically significant decrease in the total number of cortical cells in G0 (Figure 6B) and a corresponding significant increase in the number of neuroblasts in the DNA synthesis (S)-phase (Figure 6C). At 24 hours there was not a statistically significant change in the numbers of cells progressing through G2/M (Figure 6D), suggesting that estrogen-stimulated cells may have not yet completed cell cycle. There was also a non-statistically-significant trend towards a decrease in the total number of neuronal precursors that were apoptotic (Figure 6E). Our previous data also showed that estrogen decreased the expression of another member of the Fas family, the pan-neurotrophin receptor $p75^{NTR}$ [48,49]. We therefore also examined estrogen regulation of cell-surface Fas expression at different stages of cell cycle.

As observed earlier, in adherent embryonic cortical cultures, most Fas expressing cells belong to the Fas^{Mod} category and very few Fas^{Hi}-type cells can be observed (Figure 7A, control). Twenty-four hours of exposure to 2 nM E2 led to a statistically significant, 1.5-fold decrease in the percentage of cells in G0/G1 that expressed Fas (Figure 7A:E2 and 7B). In contrast, the numbers of Fas expressing cells in cell cycle, S/G2/M, were not altered by estrogen exposure (Figure 7C), though estrogen led to a statistically significant 2-fold decrease in the intensity of cell-surface Fas expression (on a per-cell basis, Figure 7D). Additionally, estrogen led to a statistically significant, 2-fold decrease in the numbers of Fas-expressing cells that were apoptotic, suggesting that estrogen suppresses apoptosis in Fas-expressing cells (Figure 7E).

Since Fas expression was also associated with p53 activation, we hypothesized that estrogen would suppress Fas expression in the context of p53 activation as well. We therefore examined the expression of Fas in *ts*TA-CHB50 cortical neuroblasts cultured for 24 and 72 hours under +*p*p53 (-*ts*TA) conditions, and treated for those durations with either estradiol-17β (2 nM) alone or estradiol-17β with the antagonist 4-hydroxytamoxifen (at 1 uM, Sigma). Our previous western immunoblot analyses

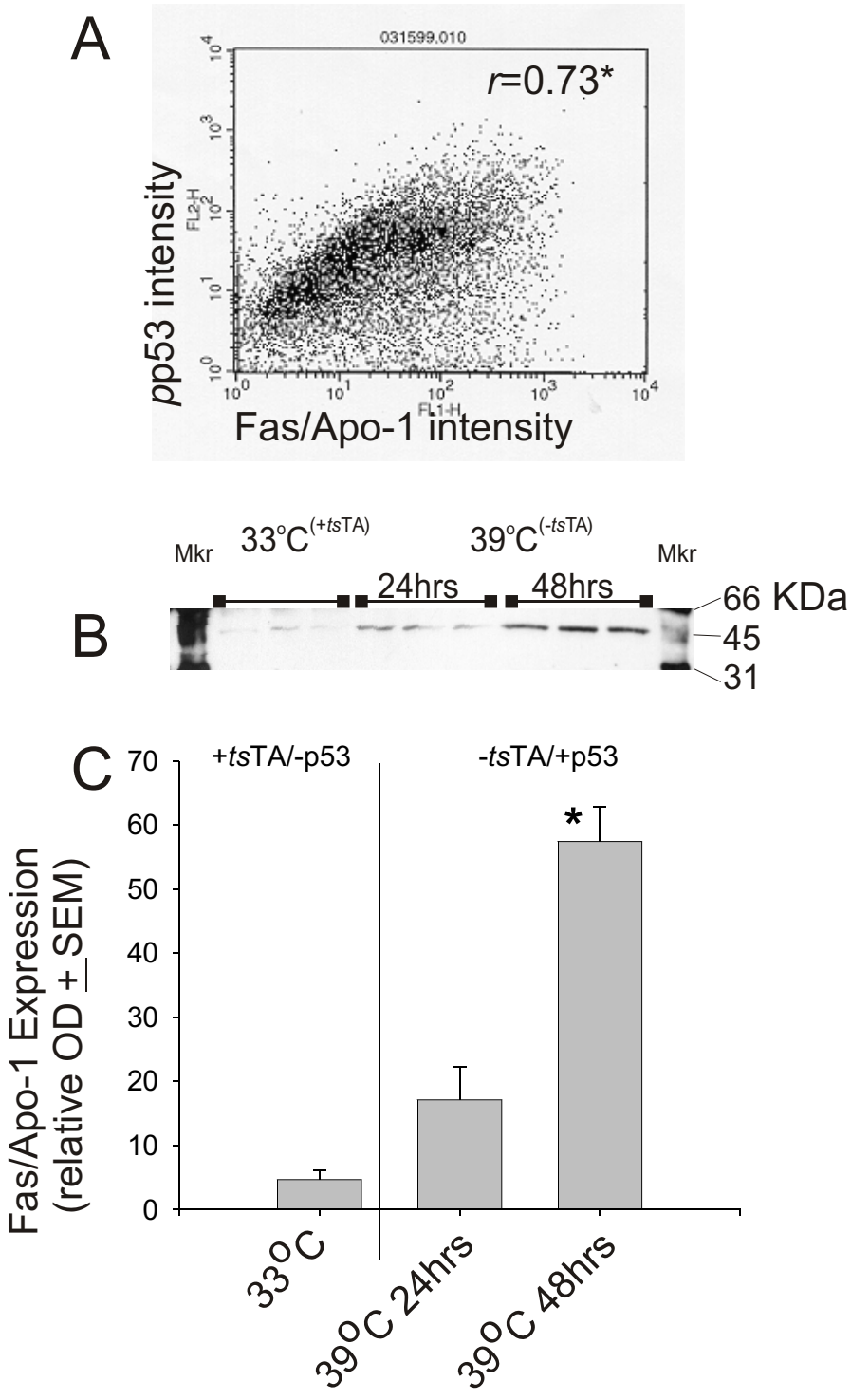

Figure 5
Fas expression is associated with p53 activation. (A) Sample flow cytometric scatter-plot showing a strong positive association between the intensity of p53 phosphorylation (*p*p53) and cell surface Fas expression in primary cortical precursors. Asterisk indicates statistical significance of Pearson's product moment correlation (inset). (B,C) Sample western blot (B) and quantitative densitometric analysis of Fas expression in conditionally immortalized CHB50 cerebral cortical neuroblasts under -p53 conditions (+tsTA/-*p*p53) and under +p53 conditions (-tsTA/+*p*p53) for 24 or 48 hours.

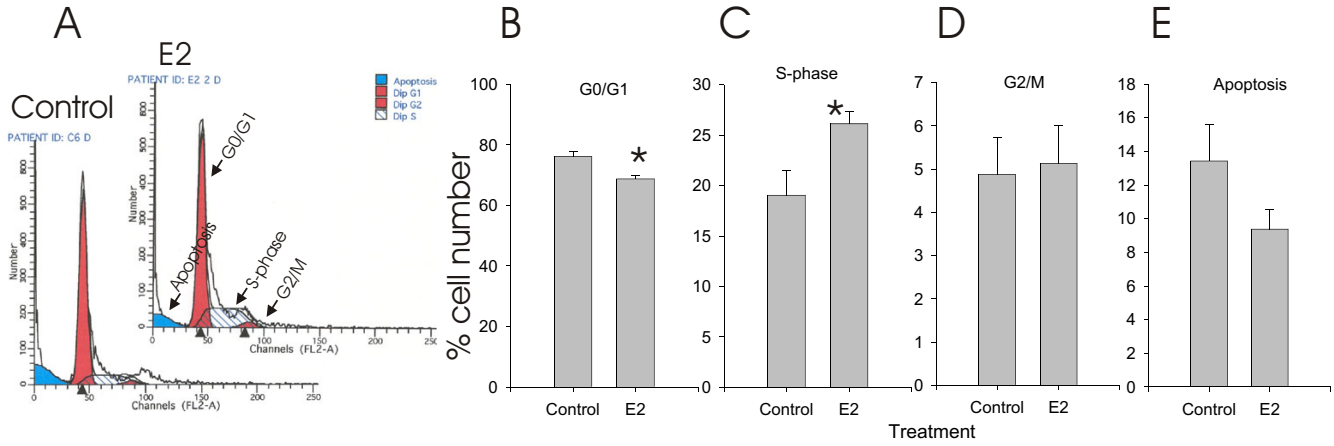

Figure 6

Estrogen promotes cell cycle in adherent primary cortical precursors: (A) Sample frequency (y-axis) distributions of PI intensity (x-axis) along with the best-fit distributions of cells in apoptosis, G0/G1, S-phase or G2/M. The area under the curve delineated by diagonal lines (S-phase) is greater in estrogen (E2)-treated cultures compared to controls. (B-E) Quantitative analysis of control and E2 treated cultures showing the mean % of cells ± SEM in G0 (B), S-phase (C), G2/M (D) or apoptosis (E). Asterisks indicate statistical significance at $p < 0.05$.

indicated that in control CHB50 cortical cultures, Fas expression was statistically significantly induced 72 hours following p53 activation (Figure 5B,5C). In the context of p53 activation, estrogen led to a significant decrease in the expression of Fas at 72 hours but not at 24 hours (Figure 8). Surprisingly (see discussion), concurrent exposure to the estrogenic antagonist 4-hydroxytamoxifen did not prevent the estrogen-induced reduction in Fas.

Estrogen and Fas regulation of the cell cycle-associated proteins

Flow cytometric analysis of primary cortical cultures indicated that estrogen led to an increase in the number of cells in S-phase (Figure 6A,6C), consistent with our previously published observations that estrogen promotes neurogenesis in cortical neuroblasts [37]. Furthermore, estrogen prevented apoptosis in cortical neuroblasts. We therefore hypothesized that in the context of Fas activation, estrogen would alter the expression of specific cell-cycle related proteins such as Cyclin-A [50], the Proliferating Cell Nuclear Antigen (PCNA, [51,52]) and p21/waf-1 [53], that also serve anti-apoptotic and DNA repair functions in addition to their roles in cell cycle. For example, PCNA and p21/Waf-1 bind to each other to prevent apoptosis induction [51], and to promote repair of double strand breaks in DNA, a characteristic of apoptosis. We therefore examined if Fas activation and estrogen could interact to regulate the expression of cyclin A, PCNA and p21/waf-1 and compared the expression of these anti-apoptotic cell cycle proteins with other cell-cycle proteins.

Dissociated embryonic cortical cells were exposed to soluble Fas ligand (sFasL, 5 ng/ml, Alexius Corp) or estradiol-17β (2 nM) either alone or concurrently, for 12 hours. Western immunoblot analysis indicated that neither estrogen nor FasL alone led to a change in the expression of PCNA. However, concurrent exposure to both estrogen and FasL did lead to a significant decrease in PCNA expression (Figure 9). Since p21/Waf-1 may prevent apoptosis by interacting with PCNA [51], we examined the expression of p21/Waf-1 in dissociated embryonic cortical cells that were exposed to sFasL, estrogen or both sFasL and estrogen for 12 hours. Neither estrogen, nor sFasL alone induced an alteration in p21/Waf1 expression, though sFasL-treated cultures exhibited a non-statistically significant trend towards decreased p21/waf-1 expression. However, the concurrent administration of estrogen and sFasL led to a significant decrease in the expression of p21/Waf-1 (Figure 9).

Cyclin-A prevents apoptosis by disrupting the formation of a complex between the E2F1 and p53 transcription factors [50]. Therefore, we examined the expression of cyclin-A following exposure to E2 and/or FasL. Both estrogen and sFasL alone led to a statistically significant increase in the expression of cyclin-A compared with controls at 12 hours post-treatment. Concurrent exposure to sFasL and estrogen was not different from exposure to either agent alone (Figure 9).

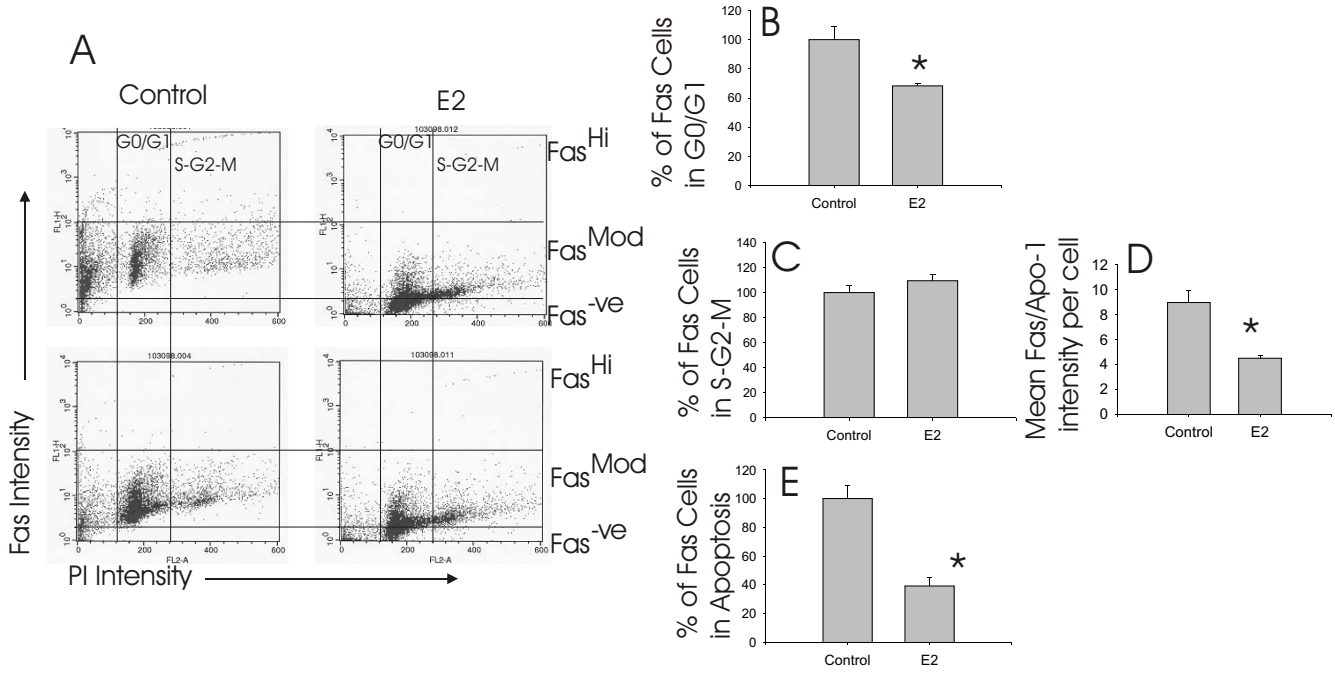

Figure 7

Estrogen suppresses Fas expression in adherent primary cortical precursors: (A) Sample flow cytometric scatter plots from two control and two E2 treated cultures, showing Fas intensity (y-axis) plotted against cell-cycle stage (PI intensity, x-axis). Scatter plots depict a general suppression in cell surface Fas intensity in E2 treated cultures compared to controls. (B,C,E) Quantitative analysis (mean ± SEM) of the number of cells expressing Fas (Fas^{Mod} + Fas^{Hi}) in control and E2 treated cultures, normalized to controls. Since these analyses were performed on adherent cells, the Fas^{Hi} population was insignificant. (D) Quantitative analysis of the mean cell-surface intensity of Fas expression in control and E2 treated cultures. Asterisks indicate statistical significance at $p < 0.05$.

Estrogen and Fas regulation of other cell cycle-related proteins

Neither estradiol-17β nor sFasL alone or together altered the expression of cdk2 or cyclin E compared to control cultures (Figure 9). Similarly, neither estrogen nor sFasL significantly altered the expression of Cyclin-dependent Kinase-1 (cdk1/cdc2) relative to control cultures, suggesting that that interactions between estrogen and Fas activation were not specifically related to cell cycle.

Discussion

The perinatal cerebral growth spurt is maintained by several competing biological processes including neurogenesis, neuronal migration, differentiation and cell death, and hence, represents a critical period of vulnerability to signals that disrupt cortical development. Cues that alter the expression of suicide receptors like Fas/Apo-1 are likely to elicit profound and enduring alterations in the organization of the developing brain. Our previous data indicate that Fas-mediated, caspase-dependent death mechanisms are present during this critical period of cortical development [9]. We therefore set out to ask the following two questions: (1) 'what developmentally relevant cell biological processes predict cell-surface expression of Fas?' and (2): 'what are the consequences of Fas activation for some of these processes?' The data reported here indicate that there are at least two subpopulations of cortical neuronal precursors that express Fas receptors, a small population that expresses extremely high levels of receptor on their cell surface (the Fas^{Hi} group) and the majority of Fas-expressing cells that express moderate levels of Fas on their cell surface (the Fas^{Mod} group). Since ventricular-zone cortical neuroblasts also express the Fas ligand [9], Fas^{Hi} neuronal precursors may be particularly sensitive to local suicide signals, and it is not surprising that, at any given time, a majority of cells in the Fas^{Hi} group are apoptotic. In culture, the Fas^{Hi} and Fas^{Mod} groups together account for approximately one third of all cells.

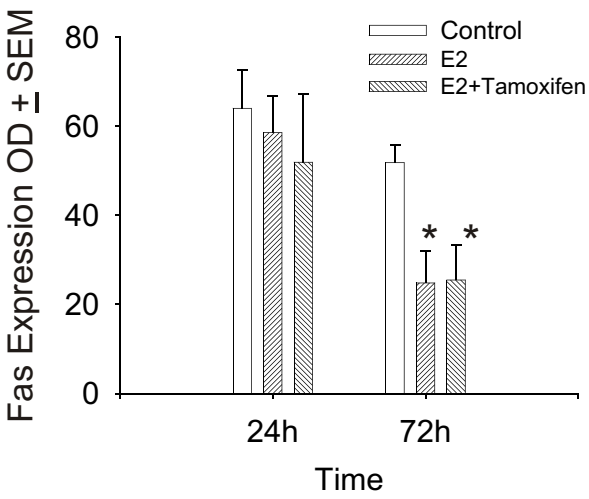

Figure 8
Densitometric analyses of western immunoblots of samples obtained from conditionally immortalized CHB50 cortical neuroblasts cultured for 24 or 72 hours following p53 induction (-*ts*TA/+*p*p53 condition). During this duration, cultures were maintained either under control conditions or were exposed to E2 alone or E2 with tamoxifen. E2 induced a significant decrease in Fas that was not reversed by concurrent exposure to tamoxifen. Asterisks indicate statistical significance at $p < 0.05$.

An analysis of our data shows that a majority (though not all) of Fas-expressing precursors express the adapter protein FADD, which enables the activation of caspase-8-dependent death [42,43]. Presumably, the non-FADD expressing neuroblasts (~27% of Fas-expressing cells) couple Fas to other death mechanisms, such as RIP [9] or DAXX [54]. Furthermore, only a small proportion of Fas-expressing cells also express the protein FLIP that is capable of blocking Fas activation [45]. We do not as yet know whether FLIP expression defines a phenotypically distinct sub-population of neural precursors. However, it is likely that this subpopulation is at least functionally distinct, since neural precursors that are resistant to suicide receptor-induced death may contribute disproportionately to the overall number of neurons in the cortical plate.

Our data indicate that cell-surface Fas/Apo-1 expression is responsive to stage of cell cycle. Though a majority of Fas^{Mod} precursors were in G0, cortical cells express higher levels of Fas (determined by immunofluorescence intensity) in cell cycle, than in G0. Concurrent-analysis of BrdU incorporation indicates that Fas-expression is particularly high in cells that have recently completed S-phase or their final round of mitosis. One possible interpretation of these data is that Fas expression, and consequently, vulnerability to Fas-induced cell death, is tied to cell cycle checkpoints. Thus, the Fas-suicide system may play a regulatory role in eliminating cortical progenitor cells that exhibit signs of genomic instability, cell senescence, errors in chromosome replication, or DNA damage. Our observation of a positive relationship between the activation of p53 (p53 phosphorylation) and Fas expression is particularly germane to the issue of cell cycle control, since p53 regulates progression through cell-cycle checkpoints [55,56]. Presumably, a p53-associated increase in cell-surface Fas expression would allow 'sentinel' Fas-ligand expressing cells in the local ventricular zone environment to eliminate damaged neuroblasts from the pool of progenitors, thereby limiting the likelihood that aberrant neurons will be added to the cortical plate.

An interesting phenomenon related to our observation that cell-surface Fas expression is highest at the end of S-phase, is the association between cell cycle and oscillatory, interkinetic movements of progenitor cells within the ventricular zone (see Figure 10, ventricular zone model). As progenitor cells transition from S-phase to mitosis, their nuclei migrate from the outer margin of the neuroepithelium to the ventricular surface [57]. Our data suggest that during this ventricular-fugal nuclear migration (post-S-phase), cortical progenitors would exhibit the highest sensitivity to Fas-induced apoptosis. This scenario is likely to be biologically important, because genetic defects in proteins such as lissencephaly-1 (LIS1) that control interkinetic nuclear movements, lead to enhanced cell death of ventricular-zone neuroblasts [58]. LIS1 regulates interkinetic movements by interacting with the microtubule-dynein motor system (reviewed in [59]). Interestingly, de-polymerization of microtubules protects hepatocytes from Fas-induced apoptosis [60]. Together, these data suggest a hypothesis that Fas expression and consequently, suicide-sensitivity, is linked to the interkinetic movements that accompany cell cycle in the cortical neuroepithelium.

Motility is also an important feature of the post-mitotic cortical neuroblast. Differentiating cortical neuroblasts exit the ventricular zone and populate the cortical plate in an inside-out gradient, with the younger neuroblasts populating successively superficial laminae [61]. Migration into the cortical plate is mediated by the interactions of receptors like the integrins with the extracellular matrix (reviewed in [59]). Our initial observation was that non-adherent neural cells predominantly segregated to the Fas^{Hi} category of cell-surface Fas-expressing cells. Therefore, in a subsequent experiment, we manipulated the interactions of neural cells with the extra-cellular matrix.

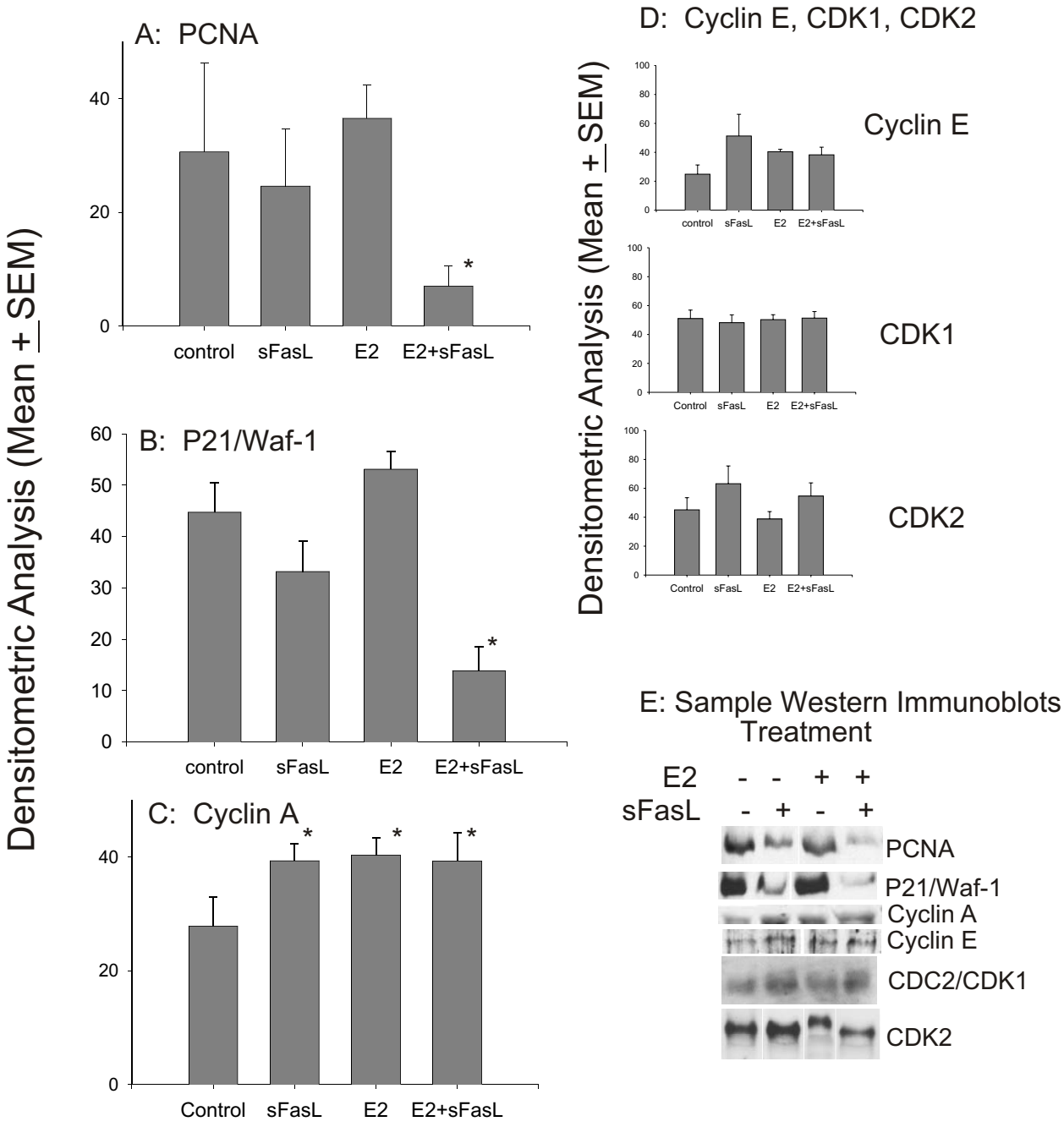

Figure 9

Estrogen (E2) and sFasL cooperate to regulate cell-cycle proteins. Densitometric analyses of western immunoblots of primary embryonic precursors treated with sFasL or E2 alone or sFasL concurrently with E2. Concurrent exposure sFasL and E2 suppress PCNA (A) and p21/Waf-1 (B) expression at 12 hours. sFasL and E2 separately and together induce cyclin-A (C). sFasL and E2 either alone or together do not regulate the expression of cyclin-E, CDK1/CDC2 or CDK2 (D). (E) Sample western immunoblots showing regulation of the expression of cell-cycle proteins following treatment with sFasL or E2 alone or together. p21Waf-1, cyclin-E and CDK-2 images were each cropped from one single immunoblot to eliminate non-relevant treatment conditions. Asterisks indicate statistical significance at $p < 0.05$.

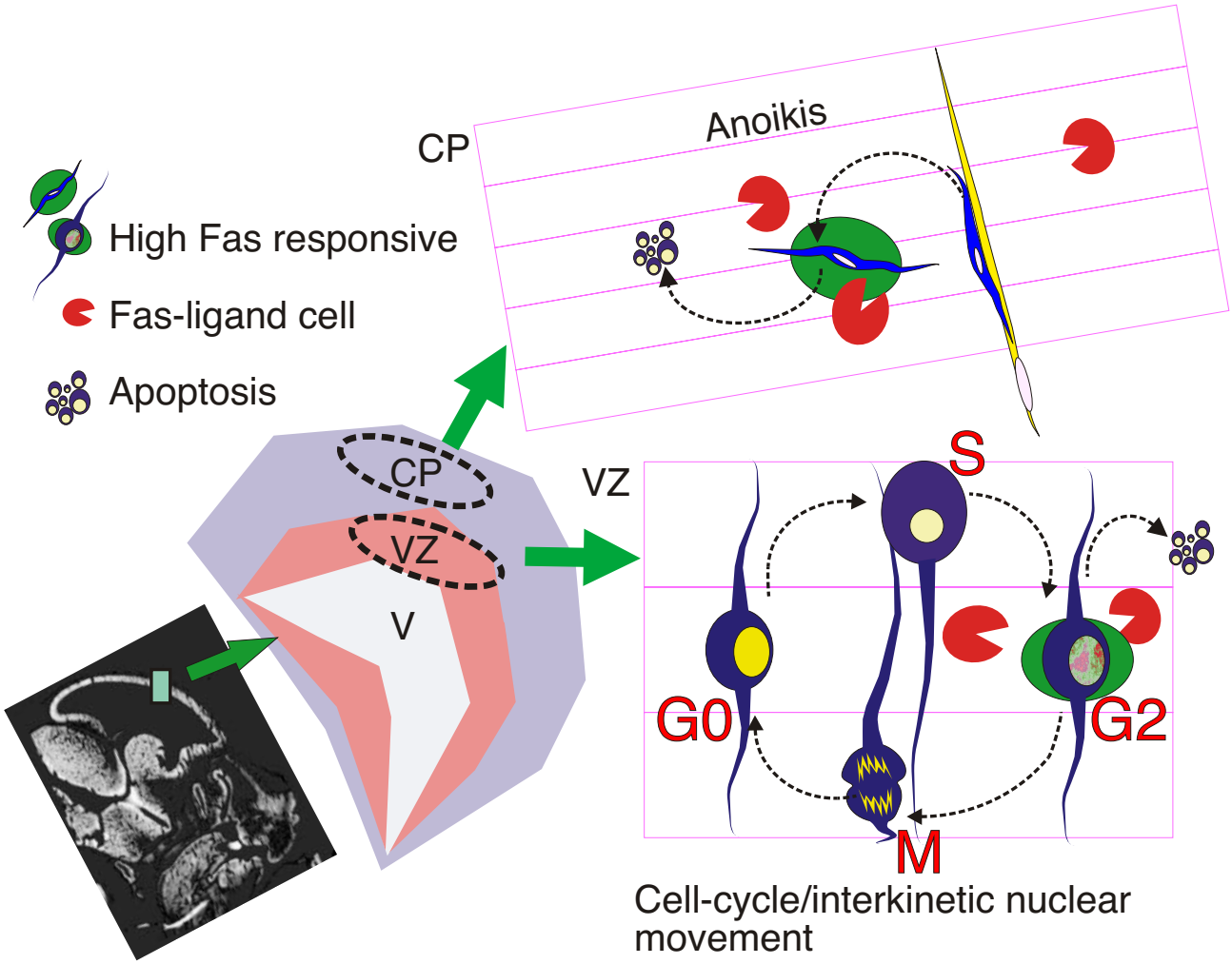

Figure 10
Models of Fas-mediated suicide sensitivity of precursors during cell cycle, and following disruption of cell-matrix interactions. *Ventricular zone (VZ)*, *interkinetic nuclear movement model*: Our data shows that during cell cycle, cell-surface Fas expression is highest in neuroblasts that also exhibit the highest level of BrdU incorporation. Such a relationship would occur at the end of S-phase, perhaps reflecting DNA replication errors. Therefore, Fas expression (indicated in the cartoon by a green peri-cellular halo), and hence suicide-sensitivity would be highest during the ventricular-fugal interkinetic movement of nuclei transitioning through G2. Resident Fas-ligand expressing cells (indicated by pacman figures) could eliminate defective Fas-expressing neuroblasts. *Cortical plate (CP)*, '*anoikis*' *model*: Cortical neuroblasts utilize integrin-mediated signals to migrate along radial glia and into the laminae of the cortical plate [98]. Collagenase-A disrupts integrin-collagen interactions, and our data shows that collagenase-A leads to increased cell-surface Fas expression. Therefore, the induction of the Fas receptor may underlie the process of 'anoikis'. 'Anoikis' in turn, may protect the developing cerebral cortex from migration errors. Abbreviations: V = ventricular zone, VZ = ventricular zone, CP = cortical plate.

Since type-1 collagen engages integrins, we used Collagenase-A to disrupt neuroblast interactions with the extracellular collagen matrix. Our results showed that loss of contact with the collagen matrix resulted in a significant increase in Fas expression, and was associated with the phenomenon of 'anoikis'. These data are consistent with a recent report showing that integrin-mediated T-lymphocyte adhesion to extracellular matrix resulted in the suppression of Fas expression [62]. Anoikis may be relevant to brain development as well, since suppression of matrix reorganization by deletion of the MMP-9 gene, leads to delayed migration of cerebellar granule cells and

decreased apoptosis [47]. Interestingly, in the context of our data on estrogen suppression of Fas expression, homozygous estrogen receptor-beta knockout animals exhibit defects in the organization of radial glia and increased apoptosis in cortical ventricular zone neuroblasts [63]. These data suggest that migration and apoptosis are contextually linked to each other *in vivo*. Perhaps, differentiating neurons that migrate to inappropriate positions within the cortical plate are eliminated by receptor-mediated suicide mechanisms (see Figure 10, cortical plate model), analogous to the mechanism that is used to eliminate self-antigen recognizing lymphocytes in the immune system [12].

The developing brain's hormonal environment has an important impact on cell fate determination and the hormone estrogen is an important regulator of cell cycle and cell suicide in cerebral cortical neuroblasts [37]. Both identified estrogen receptor subtypes, ERα and ERβ, are ligand-activated transcription factors [64-68]. Nuclear estrogen receptors and ERα mRNA are transiently expressed, at high levels in the developing cerebral cortex during the peak of neurogenesis and apoptosis [69-71]. ERβ is also expressed in the cerebral cortex [68,72,73].

Estrogen has divergent, region-specific actions on the survival of neural tissues. For example, estrogen metabolites induce apoptosis in neuroblastoma cells [74], while estrogen promotes survival in hypothalamic cell lines [75] and in the bed nucleus of Stria Terminalis [10]. Estrogen also inhibits apoptosis in the sexually dimorphic nucleus of the preoptic area while inducing apoptosis in the anteroventral periventricular nucleus of the preoptic area [76]. Furthermore, ERα and ERβ may also have opposing anti- and pro-apoptotic roles that are perhaps Fas-ligand dependent, as supported by evidence from hypothalamic cell lines [77]. In cerebral cortical neuroblasts, we have previously shown that estrogen promotes activation of the transcription factor p53, but uncouples p53 from downstream cell suicide signals to promote neuronal survival [37]. We therefore reasoned that estrogen suppresses apoptosis in the developing cerebral cortex, in part, by decreasing the cell-surface expression of the Fas receptor. This hypothesis was verified by our experiments. The numbers of neuroblasts in G0 expressing the Fas receptor declined significantly, following exposure to estradiol-17beta. Though estrogen did not alter the numbers of cells in cell cycle (S/G2/M) that expressed Fas, estrogen did lead to a significant decrease in the average intensity of Fas expression at the cell-surface. The estrogen-mediated reduction in numbers of Fas-expressing neuroblasts in G0 is open to several interpretations. The most parsimonious explanation for the data is that estrogen directly decreased Fas expression. Alternatively, since estrogen also promotes neuroblast proliferation [37], estrogen could have led to the expansion of a progenitor pool that did not express Fas on the cell surface.

We had previously observed that estrogen prevented apoptosis in T-antigen-synchronized cultures, following p53 activation (as indicated by p53 phosphorylation at serine 392, and activation of prototypic p53-responsive genes like MDM2, p21/Waf-1 and Bax) [37]. In the current study on non-synchronized primary cortical neuroblasts, we observed a non-statistically significant trend towards an estrogen-mediated decrease in apoptosis as reflected by the presence of cells with less than G0 DNA content. However, when we restricted the analysis to the Fas population, we found that estrogen induced a statistically significant decrease in the numbers of Fas-expressing cells that were apoptotic. Since Fas expression was positively associated with p53 phosphorylation, it is likely that estrogen protection against apoptosis is contextual, i.e., dependent on the activation of p53. Thus, following p53 activation in our CHB50 cortical progenitors, we found that estrogen also led to a significant decrease in Fas expression. Interestingly, the estrogen receptor antagonist, 4-hydroxytamoxifen, did not block estrogen suppression of Fas expression. We previously observed a similar tamoxifen-independent effect of estrogen on the regulation of p53 itself [37], suggesting that estrogen may regulate cell-surface Fas expression by non-classical mechanisms including transcriptional activation mediated by AP-1 [78] or activation of protein kinase pathways [79,80].

One issue that remains to be addressed is whether estrogen suppresses cell-surface Fas expression by acting as a non-classical transcription repressor, or by suppressing translocation of Fas from intracellular compartments to the cell-surface in a manner that is antagonistic to that observed for p53 [34]. The timeframe of estrogen's actions is certainly more consistent with transcription repression, and perhaps, an antagonism of p53-mediated transcription. Both the mouse and human Fas genes contain a p53 response element (p53RE) within intron #1 that drives transcription of the Fas gene [33]. *In silico* analysis of the murine Fas gene (GenBank Accession #AF282865) also indicates a potential estrogen response element (ERE) in intron#1. This presumptive ERE consists of a perfect palindrome whose halves are separated by an integer multiple of 3 random base-pairs GGTCA$(NNN)_{83}$TGACC (where the canonical ERE is GGTCA(NNN)TGACC [81]). Four additional hemi-palindromic sequences, identical to half a canonical ERE are dispersed throughout intron#1, and may also contribute to estrogen repression of Fas. These possibilities are supported by our previous research showing that intronic EREs (e.g., in the Brain-derived neurotrophic factor or BDNF gene) are functional and can contain integer multiples of the canonical triplet base-pair

spacer, between two adjacent hemi-palindromes [82]. Furthermore, repressor functions have been ascribed to estrogen receptors acting at the promoters for IGF-1 and IGF-1 receptor in aortic smooth muscle cells [83] and specific residues on ERα recruit co-repressor protein complexes [84]. One hypothesis that remains to be tested, is that estrogen receptors and p53 may be mutually repressive. While our data indicates that estrogen suppresses the expression of p53-induced proteins like Bax [37] and Fas, other data indicates that p53 can suppress estrogen receptor activation [85], by preventing ER-alpha binding to EREs.

Since estrogen induces neuroblasts to enter S-phase of cell cycle, while suppressing the cell-surface expression of Fas, we decided to examine the extent to which estrogen regulates cell cycle proteins concurrent with Fas activation. We were particularly interested in the regulation of dual-purpose proteins like cyclin-A, p21/waf-1 and PCNA that not only regulate progression through cell cycle, but also participate in DNA repair and blockage of apoptosis. For example PCNA and p21/waf-1 initiate repair of double-strand DNA breaks (a hallmark of apoptosis) [51] while cyclin A prevents apoptosis by disrupting p53 signaling [50]. Unexpectedly, concurrent exposure to both estrogen and to sFasL led to a decrease in the expression of both PCNA and p21/waf1. Since these proteins play antagonistic roles during cell cycle, it is likely that this co-regulation will have little net effect on cell cycle *per se*. On the other hand, it is likely that capacity to repair double stranded DNA breaks is curtailed. It is unclear at this time why the coordinate activation of a suicide receptor and a hormone receptor would decrease both PCNA and p21/waf1, potentially compromising DNA repair capacity. Perhaps, once cell suicide is initiated, and caspase-activated DNAses create double strand breaks, DNA repair would be an inefficient mechanism to reverse apoptosis. In this context, the role of estrogen may be to cleanly facilitate suicide. This hypothesis is consistent with observations from other laboratories [76,77] that estrogen can induce apoptosis in specific brain regions. Estrogen's anti-apoptotic role may be to prevent the initiation of apoptosis in the first place, by suppressing expression of p53-activated proteins like Fas (as reported above) and Bax [37] or alternatively, antagonizing p53 more directly. The latter hypothesis is supported by our observation that estrogen and Fas-ligand both separately and together, significantly increased Cyclin-A. Cyclin-A is a G1/S-phase cyclin, so it is not surprising that estrogen would up-regulate this cyclin as a consequence of increasing the number of neuroblasts that enter S-phase. However, cyclin-A can also antagonize p53-dependent apoptosis [50] by preventing the association between p53 and E2F, and therefore, may represent the preferred intermediary for estrogen suppression of apoptosis. Interestingly, cyclin-E, which does not compete with E2F for binding to p53 [50], was not induced by estrogen in our experiments. Additionally, cyclin-A complexes with CDK2 to induce phosphorylation of ER-alpha on serines 104 and 106, and consequent transactivation [86]. It is likely therefore, that the induction of cyclin-A increases activation of associated kinases, even though CDK2 levels were not altered, leading to feedback activation of the estrogen receptor, further suppression of Fas expression, and neuro-protection. If cyclin-A represents a means for limiting apoptosis, then the induction of cyclin A by sFasL, suggests that neuronal precursors may initiate adaptive anti-apoptotic mechanisms (aside from FLIP expression), in response to the activation of a suicide receptor.

Conclusions

Overall, our results indicate that cell-surface Fas expression identifies two distinct sub-populations of cortical neuroblasts, the Fas^{Hi} and Fas^{Mod} populations, with FLIP co-expressing cells comprising perhaps a third population. Developmentally, cell-surface Fas expression is modulated by a variety of competing factors including cell-cycle stage, extracellular matrix interactions, p53 phosphorylation and hormone availability. Dynamic changes in suicide Fas-mediated sensitivity during development are likely to play a significant role in the genesis of a variety of developmental neurodegenerative diseases including the Fetal Alcohol Syndrome [87], neonatal cerebral ischemia [15], neonatal posthemorrhagic hydrocephalus [88], neonatal brain trauma [89], and genetic diseases resulting from the expansion of polyglutamine repeats [16].

Methods

Animals

Timed-pregnant rats (Sprague Dawley) were purchased from Harlan (TX). Gestational Day (GD) zero was defined as the day on which dams were sperm positive. GD-15 rat fetuses were obtained under aseptic conditions, from pregnant dams that were anesthetized with Phenobarbital (50 mg). Fetal brains were dissected out aseptically and, in all cases, care was taken to minimize any pain and discomfort to the animals.

Dissociated primary embryonic cortical cultures

The cerebral cortical mantle of GD-15 rat fetuses was dissected out under sterile conditions, and separated from overlying meningeal tissue under a microscope. We selected GD15 because this age represents the peak period for proliferation within the cortical neuroepithelium, to generate neurons of the cortical plate. At GD15, the neuroepithelium is the dominant cortical structure, though a smaller number of differentiated neurons (the cortical preplate) and radial glia are also present. Furthermore, at this age, the neuroepithelium is mainly comprised of

proliferating neuronal (rather than glial) precursors (for review see [40]). The earliest developing layer of the cortical plate (layer VI) does not form until one day later (GD16-17). Morphologically, these cortical cultures are immature and continue to proliferate *in vitro*, but over a period of 24 hours, cultured neuroblasts become increasingly polarized and exhibit growth cones and process characteristic of early differentiating neurons. Cultures were established according to our previously published protocols [9]. Briefly, neuroblasts were dispersed by trituration in 0.5% trypsin and 6.84 mM EDTA (Sigma) and were plated on collagen-coated 24-well plates in sterile culture medium (89% Dulbecco's modified eagle media [DMEM], 10% gelding serum and 1% penicillin-streptomycin). Cultures were maintained at 37°C for 24 hours and the culture medium was replaced. For initial experiments, whole cultures were assayed 24 hours later by FACS (Fluorescence-assisted cell sorting) analysis. For subsequent experiments, only adherent cells were harvested at 24 hours and fixed for FACS analysis. For some experiments, soluble protein was extracted for western immunoblot analysis. Some experimental groups were exposed to soluble Fas Ligand (sFasL 5 ng/ml, Alexis Corp.), estradiol-17β (2 nM, Sigma) or sFasL together with estradiol-17β. Control and sFasL-treated cultures were maintained at 37°C in normal culture medium for 24 hours and then exposed to the appropriate treatments. Estrogen-treated cultures were pre-treated with estradiol-17β for 24 hours and then re-fed with estrogen alone or estrogen concurrently with sFasL for 12 hours.

Immortalized cerebral cortical culture model

To examine the regulation of Fas by p53 and estrogen, we also utilized a rodent cerebral cortical cell line (CHB50) conditionally immortalized with a temperature sensitive mutation of the SV40 large T antigen (*ts*TA) that we had previously developed and characterized [37]. In the +*ts*TA condition (incubation at 33°C), neuroblasts proliferate indefinitely and p53 is inactive. However, cessation of large T antigen expression (-*ts*TA, 39°C) is accompanied by induction of the p53 phosphorylation and the induction of p53-dependent proteins, p21/Waf1, Bax and MDM2 that lead to cell cycle-arrest, suicide, and p53 inhibition/cell cycle re-entry respectively. We had previously observed that CHB50 neuroblasts express the estrogen receptor ER-α and are estrogen-responsive. Estrogen prevents apoptosis following p53 activation and induces both neurogenesis and neuronal differentiation in this cell line. CHB50 cortical neuroblasts were therefore cultured under the +*ts*TA and -*ts*TA conditions as published previously [37] to examine the p53-related expression of Fas, and under the -*ts*TA conditions to examine the estrogen regulation of Fas during neuroblast differentiation (in the context of p53 activation).

Flow cytometric analysis

Triple parameter flow cytometric analysis was used to measure immunolabeled cell surface Fas protein, BrdU incorporation (a measure of DNA incorporation) and DNA content [propidium iodide (PI) dye intercalation] as a measure of cell cycle distribution. Dissociated embryonic cortical cultures were administered a single BrdU pulse (24 hours) at the time the cultures were established. Non-adherent and adherent [harvested by administration of collagenase (Sigma, 30 units)] cells were obtained after the initial 24-hour BrdU pulse and centrifuged at 300 × g to sediment the cells. The pellet was washed with Dulbecco's Phosphate buffered saline (PBS) and fixed in 1% paraformaldehyde and re-suspended in PBS. Cells were sedimented by centrifugation between subsequent immunohistochemical steps. To detect cell-surface Fas/Apo-1, immuno-histofluorescence chemistry was performed in the absence of detergent. Following exposure to a blocking solution (in Tris-buffered saline, (TBS) pH 7.4, with 0.1%Bovine Serum Albumin, and 2% Normal Goat Serum), cell-surface Fas/Apo-1 expression was detected with a rabbit polyclonal anti-Fas antibody (Santa Cruz, 1:500), followed by biotinylated, secondary donkey anti-rabbit (Jackson Immunochemicals, 1:1000) conjugated to streptavidin-FITC. Following Fas/Apo-1 immunofluorescence, cells were permeabilized with detergent (0.3% Trition-X100) in blocking solution. BrdU incorporation was detected with a primary anti-BrdU antibody (Sigma, 1:500) followed by biotinylated, secondary horse anti-mouse (Vector, 1:1000), conjugated to streptavidin-allophycocyanin (APC, Molecular Probes, 1:500). The cells were then washed with PBS and exposed to a PI solution (see above) for 10 minutes at room temperature. BrdU-allophycocyanin, Fas-FITC and PI fluorescence was analyzed from 10^4 cells with excitation at 488 nm (argon laser) and detection of FITC fluorescence at approximately 530 nm, PI fluorescence at 585 nm and allophycocyanin at 650 nm.

In studies examining the relationship between cell surface Fas and Fas-associated protein expression, binding of the primary antibody, mouse monoclonal antibody to Fas (Transduction Laboratories, 1:500), was detected using a rat-absorbed, biotinylated horse-anti-mouse secondary antibody (Vector, 1:1000), conjugated to streptavidin-APC or rabbit polyclonal anti-Fas antibody (Santa Cruz, 1:500), followed by biotinylated, secondary donkey anti-rabbit (Jackson Immunochemicals, 1:1000) conjugated to streptavidin-FITC. Subsequently, FLIP or phosphorylated P53 was detected using rabbit polyclonal anti-FLIP (gift from Drs. Sato and Walsh, Tufts University School of Medicine; 1:500) or phospho-p53 at serine 392 (New England BioLabs, 1:500) followed by a secondary donkey anti-rabbit (Jackson Immunochemicals, 1:1000) conjugated to streptavidin-FITC. FADD was detected using a

monoclonal anti-FADD antibody (Transduction Laboratories, 1:500) followed by a rat-absorbed, biotinylated horse-anti-mouse secondary antibody (Vector, 1:1000). Phosphorylation of the casein kinase II-sensitive site at serine 392 was used as a marker for activated p53. Phosphorylation at this site promotes tetramerization of p53 [90] and specificity of p53 binding to DNA [91], while regulating re-annealing of double-stranded DNA [92]. Cells were then washed with PBS and exposed to a PI solution [PBS, 0.1% triton (Sigma), 0.5 mmol EDTA, 0.05 mg/ml RNAse A and 50 mg/ml PI] for 10 minutes at room temperature. Fas-FITC and PI fluorescence was analyzed from 10,000 cells using a flow cytometer (Becton Dickinson) with excitation at 488 nm (argon laser) and detection of FITC fluorescence at approximately 530 nm and PI fluorescence at 585 nm. The specificity of all antibodies used for flow cytometric analysis was previously verified by western immunoblot analysis and/or immunohistochemical analysis [9,37]. Immunological controls were exposed to pre-immune serum in place of the appropriate primary antibody and were used to determine background immunofluorescence.

Western immunoblot analysis

The expression of cell cycle elements that regulate S-phase [proliferating cell nuclear antigen (PCNA)], the G1/S transition [cyclin dependent kinase 2 (cdk2) and cyclin E], the G2/M transition [cyclin dependent kinase (cdk1) and cyclin A], and a cell cycle arrest factor [p21/wild-type p53-activated fragment (Waf1)/Cip1 (cdk-interacting protein-1)], and Fas were verified by western immunoblot analysis of E15 cortical neuroblasts, according to our previously published protocols [9,37,93,94]. Detergent (1% SDS) soluble protein was isolated using the Trizol reagent (Invitrogen). Protein samples were size fractionated on an 8% SDS-polyacrylamide gel, and blotted onto supported nitrocellulose (Hibond-C-super, Amersham). Blots were blocked (5% milk, TBS (1.4 M NaCl, 0.2 M Tris)-0.1% Tween 20), exposed to primary antibody [mouse monoclonal anti-PCNA antibody (Calbiochem, 1:66); rabbit polyclonal anti-cyclin E (Calbiochem, 1:250); mouse monoclonal anti-cyclin A (Calbiochem, 1:100), mouse monoclonal anti-cdk2 (Transduction Laboratories, 1:500), mouse monoclonal anti-cdk1 (Calbiochem, 1:100) or mouse monoclonal anti-Fas (Transduction Labs)], washed and exposed to horse-anti-mouse secondary antibody (Vector, 1:1000) or donkey anti-rabbit (Jackson Laboratories, 1:5000), washed again and exposed to streptavidin horseradish-peroxidase conjugate (Amersham). Immunoreactive bands were detected using enzyme-linked chemiluminescense (NEN).

Data analysis

Data analyses for the experiments reported here were based on 5–9 independent samples. For FACS analysis, the incorporation of PI into cellular DNA was used to categorize cells in G0/G1 (diploid DNA content), S/G2/M (hyper-diploid DNA content) and apoptosis (sub-diploid DNA content) [95-97]. Cells were categorized, and intensities calculated using standard cell sorting software (Cell Quest, Becton Dickinson). Western immunoblots were analyzed using standard densitometric software (Molecular Analyst, Bio-Rad). Sample size for western blot analyses was 6. Statistical differences were calculated using ANOVAs followed by post-hoc tests (Student-Newman-Keuls, $p < 0.05$). Correlational analyses were calculated by combining mean intensity values from 6–8 replications using Pearson's product moment correlation (r) followed by a two-tailed test for the significance of the correlation coefficient. Levels of statistical significance were set at $p < 0.05$. Data were expressed in terms of mean ± standard error of the mean (SEM).

Abbreviations

APC = Allophycocyanin

BrdU = Bromodeoxyuridine

CDK1 = CDC2, Cyclin-dependent kinase 1

CDK2 = Cyclin-dependent kinase 2

CP = Cortical Plate

DISC = Death Inducing Signaling Complex

E2 = Estradiol-17beta

ERE = Estrogen response element

FADD = Fas-associated death domain containing protein

Fas = Fas/Apo [apoptosis]-1/CD95 suicide receptor

Fas^{Hi} = High intensity of cell surface Fas immuno-fluorescence

Fas^{Mod} = Moderate intensity of cell surface Fas immuno-fluorescence

Fas^{-ve} = Undetectable cell surface Fas immuno-fluorescence (not different from background)

FITC = Fluorescein isothiocyanate

FLIP = FLICE (caspase-8)–inhibitory protein

FU = Fluorescence Units

p21/Waf1 = Wild-type p53-activated fragment 1 or cyclin-dependent kinase inhibitor 1A

p53RE = p53 response element

PCNA = Proliferating cell nuclear antigen

PI = Propidium iodide

*p*p53 = Phosphorylated p53 (on serine 392)

SEM = Standard error of the mean

sFasL = soluble Fas-ligand

*ts*TA = temperature-sensitive SV40 T antigen

V = Ventricle

VZ = Ventricular Zone

Authors' contributions

ZFC, DRS, JMN & SBW contributed to the conduct of the experiments. ZFC & RCM contributed to the data analysis, ZFC and RCM conceived the experiments and the experimental design, ZFC, DRS and RCM wrote the manuscript.

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