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13. ABSTRACT (Maximum 200 Words)

Coactivators are nuclear proteins that interact with steroid receptors, such as estrogen receptor-α (ERα), and are required for the ability of receptors to stimulate the expression of target genes. Antiestrogen ligand are commonly utilized in the treatment of breast cancer to negatively regulate the activity of steroid receptors. However, tumors often can develop resistance to antiestrogen therapy. Therefore, as an alternative approach to inhibiting ERα function in breast cancer cells, we have developed antisense oligonucleotides against three of the major ERα coactivator proteins. These oligonucleotides decrease the expression of coactivator mRNA and protein, and in so doing, decrease the ability of ERα to stimulate gene expression. These oligonucleotides also decrease the proliferation of MCF-7 breast cancer cells in response to estrogen treatment. Taken together, antisense oligonucleotide technology has the potential to regulate ERα action at a level that circumvents ligand control, and therefore represents a novel mechanism by which to inhibit breast cancer gene expression and proliferation, and potentially to regulate the growth of breast cancer.

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Introduction

Although estrogens are important for normal breast development, they also have been linked to breast cancer, at least in part through their ability to stimulate cell proliferation. Inhibition of the ability of ER to mediate estrogen action therefore has been a major goal in the treatment, and more recently prevention of hormone-dependent breast cancer. Estrogen effects are mediated by two, high affinity intracellular receptor proteins, estrogen receptor-α (ERα) and estrogen receptor-β (ERβ), that are members of a superfamily of transcription factors. Once activated by ligands, these receptors interact with coactivators that positively enhance ER transcriptional activity by acting as bridging factors to the general transcriptional machinery as well as modifying local chromatin structure. Amongst the best characterized coactivators that interact with ER are members of the steroid receptor coactivator family of coactivators (SRC-1, SRC-2/TIF2 and SRC-3/RAC3/AIB1). Recently it was determined that AIB1 (SRC-3) was amplified in 7% of human breast tumors, and that overexpression of AIB1 correlated with increased tumor size, suggesting a important role for this coactivator in regulating breast cell growth and possibly tumorigenesis. Furthermore, targeted deletion of the gene for SRC-1 in mice results in generalized resistance to steroid hormones and underdeveloped mammary glands characterized by reduced ductal branching and lobuloaveolar development thus reinforcing the concept that coactivators are required for efficient ER-mediated biological responses *in vivo* [1]. Tamoxifen therapy is used in the prevention and more commonly, the treatment of breast cancer. Its biological effects are related to its ability to inhibit ER action. However, in advanced disease, tamoxifen therapy fails, and may even promote tumor growth. It is interesting to note that the experimental evidence to date indicates that when tamoxifen stimulates ER action, it does so in conjunction with ER coactivator proteins. Thus coactivators are a common mediator of ER action regardless of whether estrogen or tamoxifen is present, and they therefore represent a more universal target for inhibiting ER action than alternative antiestrogen (ligand-based) therapies.

Body

Year 1 Results

Antisense Oligodeoxynucleotides. Most cellular studies on the functions of coactivators and corepressors have relied upon their overexpression followed by subsequent characterization of the event of interest. While this approach has provided solid information on the potential role of these molecules in ERα action, the results may lack specificity due to overexpression. This obstacle could be overcome in cell lines lacking expression of the target of interest (*e.g.* mouse embryo fibroblasts from knock-out mice), but these cells limit the scope of biological processes that can be examined and may be influenced by compensatory overexpression of other coactivators as seen in the SRC-1 knock-out mouse [1]. Therefore, in collaboration with ISIS Pharmaceuticals, we have developed antisense oligonucleotides that significantly and specifically inhibit the expression of a series of coregulators including SRC-1, TIF2 and RAC3. This is possible because oligonucleotides of up to 20 bases are able to discriminate between two gene products that differ by a single base [2]. These oligonucleotides utilize a unique chemistry that yields a stable and effective inhibitor of target gene mRNA and protein expression. These oligonucleotides are also very specific for the target of interest. For example, in cells treated with a TIF2 antisense oligonucleotide, only TIF2 and not SRC-1 mRNA, is down regulated (**Fig.**

1A). Expression is also reduced at the protein level as demonstrated by the ability of SRC-1, but not TIF2 antisense oligonucleotides to reduce SRC-1 protein expression (**Fig. 1, C&D**). Importantly, we have shown that suppression of mRNA levels can be maintained for up to 72 hours (**Fig. 2**), and this agrees with other published reports using oligonucleotides with this unique composition [3]. We also have tested antisense oligonucleotides against RAC3, which effectively suppress its mRNA expression (**Fig. 3**). Using FITC-labeled oligonucleotides, we have demonstrated that oligonucleotides are taken up by virtually 100% of the cells (**Fig. 4**). We have also shown that oligonucleotides against SRC-1, TIF2 and SRA inhibit ERα transcriptional activity measured on a 3xERE-TATA-Luc reporter as well as coactivator expression in a dose-dependent manner in HeLa cells (**Fig. 5**).

Using antisense oligonucleotide technology, we proposed to examine the role of these specific coactivators in regulating ERα activity. We chose the MCF-7 cell as a starting point for these studies because these cells are ERα-positive, enabling us to perform experiments without having to transfect ERα expression plasmid. This also permitted an examination of the role of specific coactivators on well characterized ER-mediated events, such as induction of gene regulation or stimulation of cell growth. Our results demonstrate the impact of decreasing coactivator expression on estrogen induction of pS2 mRNA and find that antisense oligonucleotides against SRC-1 and TIF2, but not SRA inhibit pS2 gene expression (**Fig. 6A**). Parallel analyses of these same RNA samples for coactivator mRNA levels verify that the antisense oligonucleotides decrease their expression as expected (**Fig. 6B**). This contrasts with the ability of antisense oligonucleotides against each of these coactivators to inhibit expression of a transiently transfected ER target gene in HeLa cells (see above). Taken together, these results suggest the interesting possibility that the importance of specific coactivators to ERα activity may vary depending on the specific target gene (ERE-TATA-CAT *versus* pS2), whether the target is endogenous or transiently expressed, or perhaps by cell type (HeLa *versus* MCF-7 cells). With the development of these novel oligonucleotides, we are well positioned with the research tools to examine the contribution of coactivators to ligand-specific, gene-specific and cell-specific regulation of ERα function.

Effects of Coactivator Antisense Oligonucleotides on MCF-7 Cell Growth. MCF-7 cells express ERα and their growth is estrogen-dependent [4]. Therefore, in order to determine if any of the coactivators examined in our studies contributed to estrogen-mediated growth, MCF-7 cells were transfected with the indicated quantity of *as*ODNs or their corresponding *rs*ODNs, and twenty-four hours thereafter cell proliferation was assessed by [^{3}H]thymidine incorporation. It is important to note that these studies are possible because virtually all the cells uptake ODN. As shown in **Fig. 7A**, *as*ODNs to SRC-1 or TIF2 decreased cell proliferation in comparison to cells transfected with appropriate levels of *rs*ODN. Interestingly, SRA *as*ODN did not inhibit [^{3}H]thymidine incorporation in these cells, but instead had a modest stimulatory effect on DNA synthesis. These results were further substantiated in cells grown in stripped serum in the absence or presence of 1 nM E2 to ensure that the *as*ODN inhibited estrogen-induced cell proliferation (**Fig. 7B**). Estradiol stimulated [^{3}H]thymidine incorporation in SRA, SRC-1 or TIF2 *rs*ODN-treated cells by 4-5 fold. These increases in DNA synthesis were attenuated in cells treated with *as*ODNs to either SRC-1 or TIF2, but not to SRA; a result similar to that obtained for Fig. 7A.

Cell Models of Selective 4HT's Agonist/Antagonist Activity. We have obtained MCF7/LCC1 and MCF7/LCC2 cells from Dr. Robert Clarke at the Lombardi Cancer Research Center (Georgetown University). As will be explained below, these cells are derived from MCF-7 (human breast) cells, which require estrogen for their growth and are growth inhibited by 4HT. The derivative cell lines, which will be referred to as LCC1 and LCC2, have significant alterations in their growth responses to E2 and 4HT [5;6]. However, since these cells are from a common lineage, the relative differences between them should be biologically, and will provide information about alternations in the expression and activity of coactivators and their functions that occur as breast cancer cells progress to a hormone-independent, but hormone-responsive phenotype.

The LCC1 cells were derived from MCF-7 cells that had been passaged twice in ovariectomized athymic nude mice [5]. These cells do not require E2 for growth in culture and are therefore considered to be hormone-independent [5;6]. However, like the MCF-7 parental line, they are growth inhibited by 4HT as well as the pure antiestrogens, ICI 164,384 and ICI 182,780 [5;7]. The LCC1 cell line also exhibits a more malignant phenotype that has been attributed to altered gene regulation rather than gene amplification [5]. Stepwise selection of LCC1 cells in increasing concentrations of 4HT produced the LCC2 cell line which grows independently of estrogen and is resistant to growth inhibition by 4HT *in vitro* and *in vivo* [6]. Importantly, these cells also retain sensitivity to ICI 182,780 and ICI 164,384 [6;8] indicating that they still possess functional ER responses, and that this resistance is specific to 4HT (**Fig. 8**). None of these cells express detectable aromatase activity [9], but all express equivalent levels of ER [5]. In each cell line, the E2-induction of pS2 gene expression by estrogen, considered to be a primary estrogenic response [10] and cathepsin D is retained [5;8;11]. We are now prepared to examine the impact of decreasing coactivator expression in these cell lines.

Year 2 Results

Having established that the MCF-7, LCC1 and LCC2 cell lines exhibit different cell growth characteristics to estrogen and antiestrogen, we began our analyses of coactivator expression in these cells lines. It has been demonstrated in the literature that differences in the expression of SRC-1 between two different cell lines may be responsible for their different responses to tamoxifen [12]. Specifically, the Ishikawa uterine cell line has greater expression of SRC-1 than the MCF-7 breast cancer cell line, and tamoxifen is able to stimulate the expression of the c-Myc and IGF-1 genes in Ishikawa but not MCF-7 cells. However, when siRNA was used to reduce the expression of SRC-1 in Ishikawa cells, tamoxifen lost its ability to stimulate the expression of these two target genes in Ishikawa cells. Notably, siRNA to RAC3 (also known as AIB1) did not affect tamoxifen's agonist activity. To determine whether alterations in the expression of any of the p160 coactivators in the MCF-7, LCC1 and LCC2 cells could be contributing to their different responses to ER ligands (or ligand-independent activity), we assessed p160 coactivator expression by Western blot. Our first task was to identify antibodies that would perform well in these assays. A number of antibodies for these factors are commercially available. We tested a number of them and found that the GeneTex antibody for SRC-1, the Becton Dickinson antibody for TIF2 and the Becton Dickinson antibody for RAC3 worked well under our experimental conditions. Using these reagents, we found that expression of each of the p160 coactivators was expressed at comparable levels in our model cell lines (**Fig. 9**). Thus we are able to conclude that a gross alteration in the expression of any of these coregulators is not likely to contribute to their different responses to ER ligand treatment. Moreover, our finding raises the possibility that

elevated SRC-1 expression and the ability of tamoxifen to act as an agonist may hold true only for uterine cells, or may even be a response specific only to Ishikawa cells.

Our next step was to determine the efficacy with which oligonucleotides entered the breast cancer cells to be used in our study. This was accomplished by first, determining the appropriate level of Lipofectin (Invitrogen) to be used to introduce oligonucleotides into each of the cell lines. Titration studies were performed, and 4 μl of Lipofectin was found to effectively introduce oligonucleotides for each cell line. We next evaluated the uptake of fluorescent-tagged oligonucleotides in each of the cell lines. Cells were harvested at three different time points after the transfection –4, 24 or 48 hours after the addition of oligonucleotides – in order to be able to assess the duration of oligonucleotide persistence. As shown in **Figs. 10-12**, each of the cell lines takes up oligonucleotides with very good efficiency, and retains the label for at least 48 hours. The oligonucleotides therefore appear to work as well in these breast cancer cells as they did in the HeLa cells that we initially used to characterize the oligonucleotides.

The ability of the antisense oligonucleotides was then assessed for its ability to inhibit the expression of the target mRNAs in each of the cell lines. Previously, mRNA expression for the coactivators had been assessed by Northern blot analysis. While this method provides satisfactory results, it requires relatively large amounts of material which necessitates that large numbers of cells must be transfected with a corresponding relatively large amount of oligonucleotide. Moreover, Northern blots are time consuming and require significant effort to generate quantitative results. As these studies have evolved into examining the effect of the oligonucleotides in 4 different cell lines under various ligand treatment conditions, it was important to utilize a method to be able to assess coactivator mRNA levels in a more rapid, quantitative and reagent efficient manner. We therefore now perform our RNA analyses by real time, RT-PCR reactions utilizing TaqMan technology. These assays are quantitative, require only small amounts of input RNA (< 1 μg), and can provide data on coactivator mRNA levels and 18S RNA in a matter of several hours.

Using this approach, we first determined the optimal amount of SRC-1 antisense oligonucleotide to inhibit SRC-1 mRNA expression in MCF-7 cells. We began our experiments with SRC-1 antisense oligonucleotides as the first target since this coactivator was implicated for a role in tamoxifen agonist activity [12]. A dose response experiment employing 0 to 250 pmol revealed that maximum inhibition of SRC-1 mRNA expression was achieved with 200 pmol; the addition of more oligonucleotide was without effect (**Fig. 13**). The ability of 200 pmol of the SRC-1 antisense oligonucleotide to decrease SRC-1 mRNA expression in MCF-7, LCC1 and LCC2 cells lines was then assessed and this experiment revealed that the antisense oligonucleotide against this coactivator is effective, to a similar extent, in all three cell lines (**Fig. 14**). For our next target we choose RAC3 since this coactivator is overexpressed in breast cancer cell lines in comparison to many other cell types [13]. We found that when 200 pmol of the RAC3 oligonucleotide was transfected into cells, no inhibition of RAC3 mRNA expression was observed (**Fig. 15**). This was unexpected since we previously had determined that the RAC3 antisense effectively decreased RAC3 mRNA expression in HeLa cells (Fig. 3).

siRNA has emerged as an important technology that enables the reduction of target mRNAs. Through an interaction with a long term collaborator, Dr. Bert O'Malley, we were able to obtain sequence information for siRNA for each of the p160 coactivators; the sequence of these siRNA has been recently published [14]. We therefore determined whether a siRNA designed for inhibition of RAC3 mRNA expression could effectively inhibit this target in breast cancer cells.

As shown in **Fig. 16a**, the RAC3 siRNA was effective as revealed by the ability of this reagent to reduce RAC3 mRNA expression in each of the three cell lines. Moreover, **Fig 16b** demonstrates that the siRNA also reduces extensively the expression of RAC3 protein in these cells. We have also determined that the siRNA against TIF2 mRNA was effective in all three cell lines (**Fig. 17**). Thus this alternative technology will enable us to assess the effect of depleting RAC3 or TIF2 mRNA expression on the responses of these cells to ligand stimulation. Taken together these results demonstrate that we have established the reagents and conditions for depleting p160 coactivator expression in the model breast cancer cell lines.

Having established conditions to inhibit coactivator expression, we then turned our attention to developing suitable methods for assessing the effect of coactivator depletion on ligand-regulated cell growth. As shown in **Fig. 18**, two methods have been evaluated. Direct counting of cell number was performed to be able to directly assess the number of cells present after 5 days of hormone treatment, and the results are as expected; LCC1 and LCC2 cells grow well in the absence of ligand, all three cell types grow well in the presence of E2, the LCC2 exhibits superior cell growth in the presence of 4HT, and none of the cell lines grow in the presence of ICI 182,780. Pilot studies had been performed to determine the number of days of cell growth in the presence of ligand required to be able to determine cell-type dependent differences in their responses; five was found to be the shortest period of time in which distinct differences could be observed. Previously, we had used [^{3}H]thymidine incorporation to investigate the effect of ligands on MCF-7 cell growth and the ability of E2 to stimulate that process. As shown in **Fig. 18b**, this assay revealed expected patterns of results for cells grown in the absence of ligand, or in the presence of E2 or ICI. However, the results for 4HT was initially surprising since they indicated elevated levels of DNA synthesis for both LCC1 and LCC2 cells. Since only LCC2 cells grow in the presence of 4HT (**Fig. 19**), we interpret this data to indicate the 4HT may exert its apparent growth inhibitory effects via induction of apoptosis in LCC1 cells as opposed to a direct inhibition of cell replication as indicated by DNA synthesis assay. This will be examined in more detail in future studies.

Using the conditions established above, the effect of inhibition of SRC-1 expression was determined for MCF-7 and LCC1 cells. As shown in **Fig. 20**, depleting SRC-1 expression inhibited E2-stimulated cell growth, while depleting this coactivator did not affect the growth of LCC1 cells under ligand-free or estrogen-stimulated conditions. This result will be verified and the effect of SRC-1 deletion in LCC2 cells assessed. Verification of this finding would indicate that as LCC2 cells have become independent of E2 for cell growth, that they have also become independent of SRC-1. This finding would have important implications for the development of alternative approaches to controlling the growth of breast cancer cells, as one proposal has been to regulate the function of coactivators such as SRC-1 in order to provide more effective control of ER-dependent processes.

Salary support over the course of this project was provided to Carolyn Smith and Basem Jaber.

Key Research Accomplishments

1. Development and verification of antisense oligonucleotides against the coactivators, SRC-1, TIF2 and RAC3.

2. Oligonucleotides against SRC-1, TIF2 and SRA reduce the transcriptional activity of ERα in HeLa cells.
3. Oligonucleotides against SRC-1 and TIF2 reduce the expression of the estrogen target gene, pS2 in MCF-7 cells.
4. Oligonucleotides against SRC-1 and TIF2 reduce the estrogen-dependent growth of MCF-7 cells.
5. Inhibition of cell growth and induction of pS2 expression is specific to certain coactivators, since reductions in the expression of the SRA coactivator, did not inhibit MCF-7 cell growth or estrogen induction of pS2 mRNA expression.
6. There are insufficient differences between the expression of SRC-1, TIF2 and RAC3 protein in MCF-7, LCC1 and LCC2 cells to account for the different responses these cells mount to environments deficient in estrogens or replete with the tamoxifen antiestrogen.
7. Conditions have been established that enable depletion of SRC-1, TIF2 and RAC3 expression in each of the three breast cancer cell lines.
8. Results to date indicate that depletion of SRC-1 negatively impacts MCF-7, but not LCC1 cells growth; this may indicate that these cells now circumvent the requirement for SRC-1 in the regulation or cell growth.

Reportable Outcomes

1. Funding applied for and received, based in part on this work – "Coactivator antisense oligonucleotides as therapeutic targets for breast cancer." Funded by the State of Texas Advanced Technology Program – Biomedicine (01/01/02 – 12/31/03).

Conclusions

Our work to date indicates that antisense oligonucleotides against SRC-1, TIF2 and RAC3 (SRC family coactivators) decreases the expression of the mRNAs and proteins for these targets. We have also established the antisense oligonucleotides technology in breast cancer cells lines and have also incorporated siRNA against the coactivators to ensure that we are able to effectively inhibit coactivator expression in these cells. Our results indicate that elevated SRC-1 is not necessary for the acquisition of tamoxifen resistance, and suggest that more detailed, comprehensive studies of this important biological and clinical phenomenon are required. We now have the reagents to explore the role of these coactivators in regulation of cell growth and gene expression in the presence of estrogens and antiestrogens. This clearly established these coactivators as important *in vivo* determinants of these biological processes, and advances the principle that they may be targeted for affecting breast cancer cell growth. We continue to explore this avenue of investigation.

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Appendixes

Please see figures on pages 11 to 30.

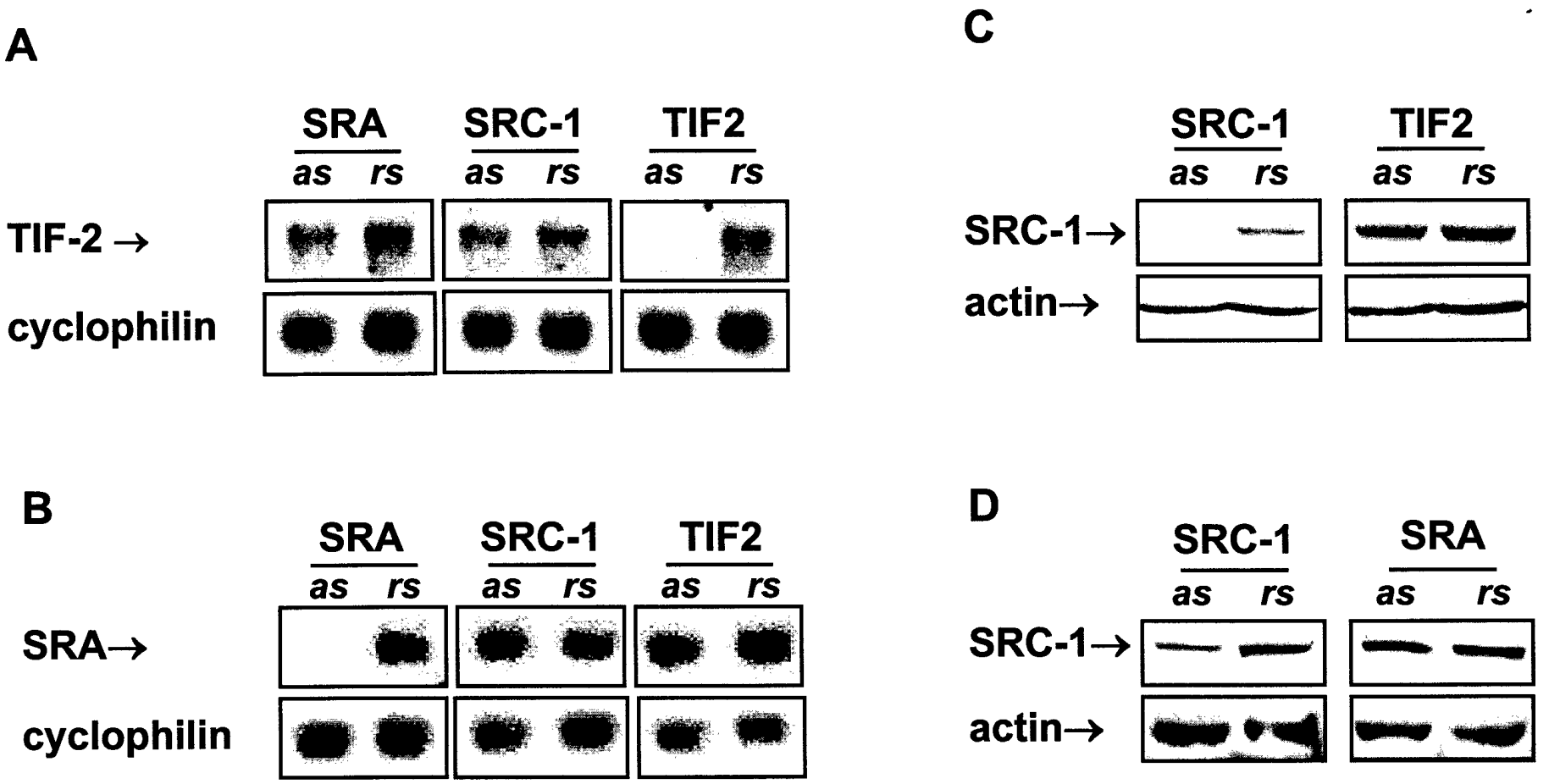


Figure 1: SRA, SRC-1 and TIF2 *as*ODNs are specific for their mRNA/protein targets. **A)** Northern blot analysis of total RNA extracted from cells treated for 4 hours with 100 pmol of *as*ODN or *rs*ODN for SRA, SRC-1 or TIF2 and harvested 24 hours later. TIF2 and cyclophilin mRNAs are indicated. **B)** Cells treated with 200 pmol of the same asODNs and rsODNs were similarly subjected to Northern analysis for SRA expression. **C)** Western analysis of proteins extracted from cells treated for 4 hours with 100 pmol of *as*ODN or *rs*ODN for SRC-1 or TIF2 and harvested 24 h later. SRC-1 and actin proteins are indicated. **D)** Western analysis of proteins extracted from cells treated for 4 hours with 50 pmol of SRC-1 *as*ODN, SRC-1 *rs*ODN, SRA *as*ODN or SRA *rs*ODN and harvested 24 h later. SRC-1 and actin proteins are indicated.

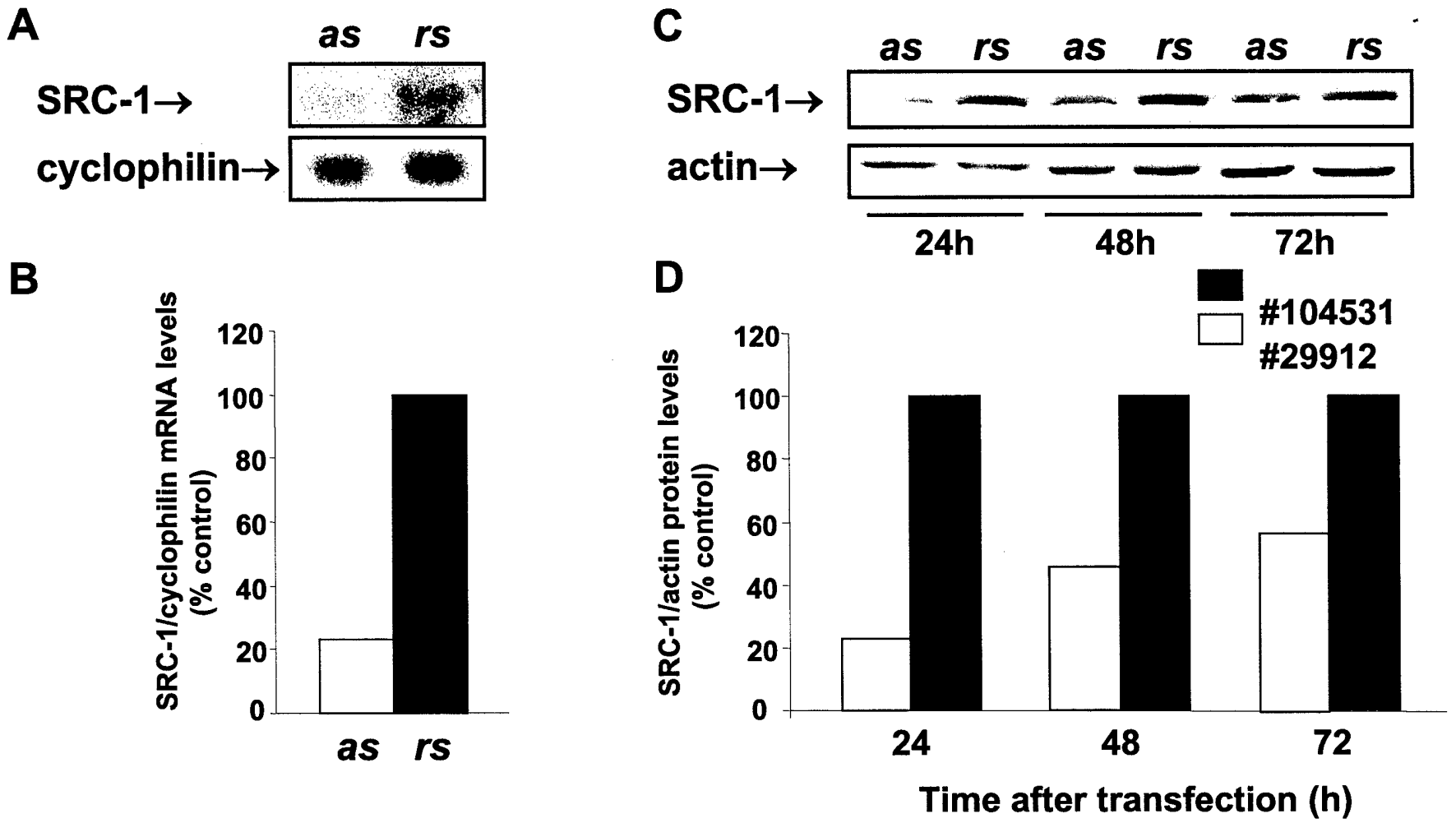


Figure 2: Effect of SRC-1 *as*ODN on SRC-1 mRNA and protein expression. **A)** Northern blot analysis of total RNA extracted from cells treated for four hours with 200 pmol of SRC-1 *as*ODN (#29912) or *rs*ODN (#104531) and harvested 24 hours later. SRC-1 and cyclophilin mRNAs are indicated. **B)** Quantification of the Northern blot by scanning laser densitometry. SRC-1 mRNA levels in the presence of the *as*ODN (open bar) are corrected to cyclophilin mRNA levels and expressed as percentage of the SRC-1 mRNA levels measured in the presence of an equivalent quantity of the corresponding *rs*ODN (solid bar). **C)** Western blot analysis of proteins extracted from cells treated with 200 pmol SRC-1 *as*ODN or *rs*ODN for four hours and harvested 24, 48 or 72h thereafter. SRC-1 and actin proteins are indicated. **D)** Quantification of the Western blot by scanning laser densitometry. SRC-1 protein levels in the presence of the *as*ODN (open bar) are corrected by actin protein levels and expressed as percentage of the protein levels measured in the presence of equivalent amounts of the corresponding *rs*ODN (solid bar).

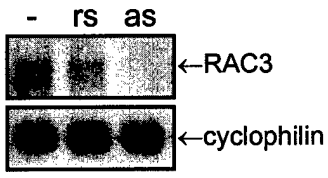


Figure 3: RAC3 antisense oligonucleotides inhibit RAC3 mRNA expression. HeLa cells were treated with 100 pmol of random or antisense (rs or as, respectively) oligonucleotides or left untreated ('-'). RNA was extracted 24 h thereafter and subjected to Northern blot analyses for RAC3 (*top*) and cyclophilin (*bottom*) mRNA levels.

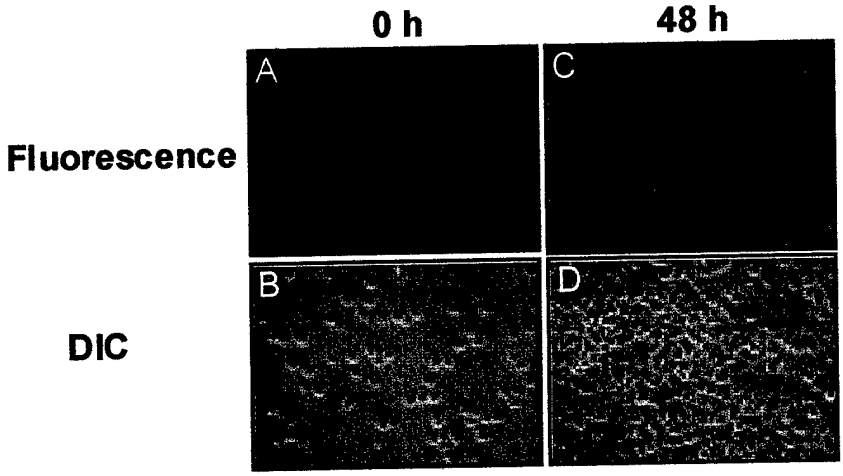


Figure 4. Uptake of a FITC-conjugated ODN by HeLa cells. Cells were transfected with a fluorescein-conjugated ODN using Lipofectamine. The efficiency of transfection was evaluated by observing the cell fluorescence pattern immediately after removing the Lipofectamine/ODN mixture (**A**) and 48 hours thereafter (**C**) and by comparing the number of fluorescent cells to the total number of cells observed by DIC-microscopy (**B and D,** respectively).

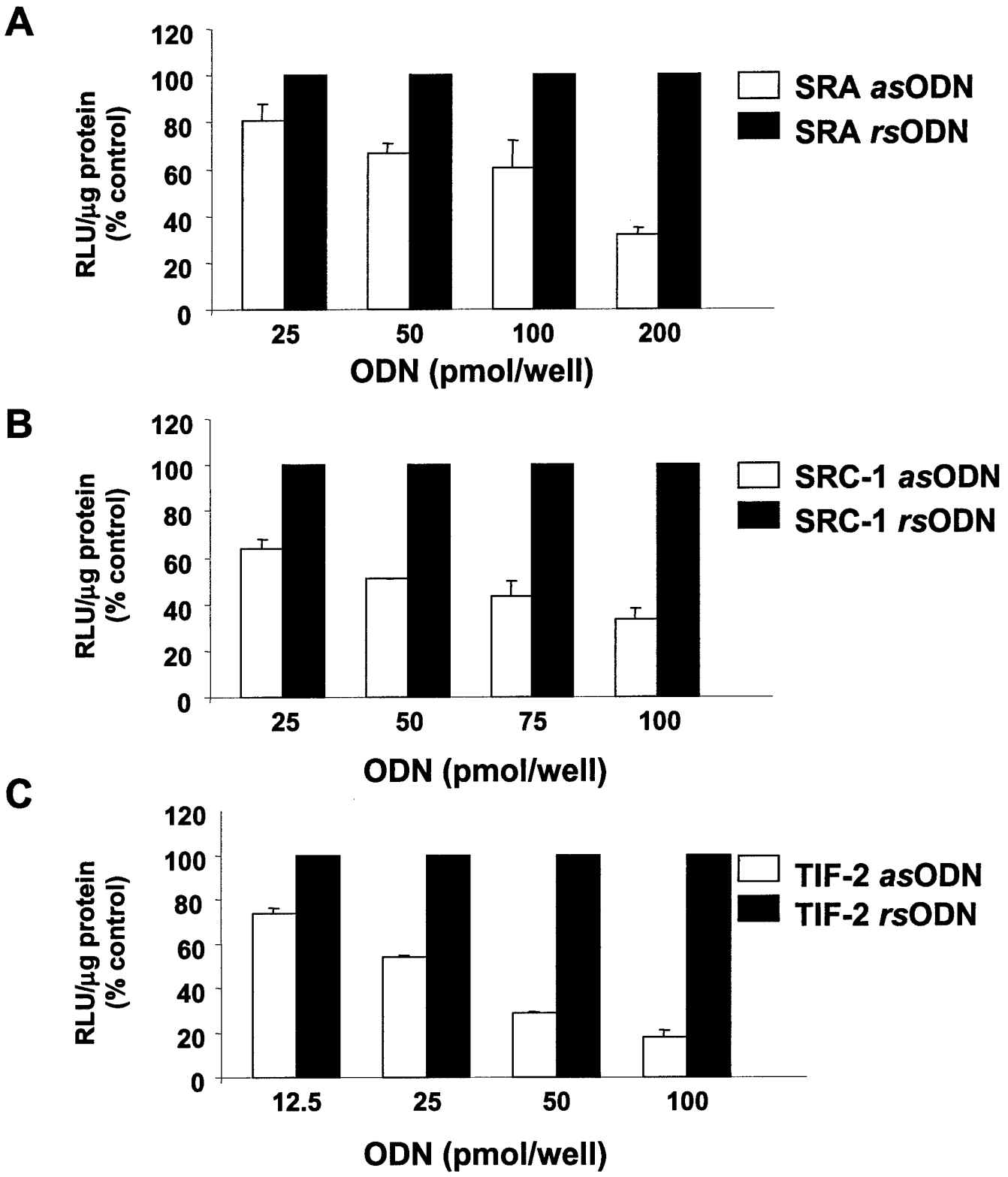


Figure 5: ERα-transcriptional activity is impaired in a dose-dependent manner by the presence of SRA, SRC-1 and TIF-2 *as*ODNs. **A)** Cells were transfected for two hours with 25, 50, 100 or 200 pmol of SRA *as*ODN (open bar) or with equivalent quantities of the corresponding *rs*ODN (solid bar) along with $pCMV_5hER\alpha$ and a 3xERE-TATA-Luciferase target gene, and treated with 1 nM estradiol. Luciferase activity represents the mean of duplicate samples obtained from cells treated with *as*ODN expressed as a percentage of the RLU from cells treated with the *rs*ODN. Each plot represents one of at least three independently repeated experiments. Values from cells treated with *as*ODN or *rs*ODN for SRC-1 and with the *as*ODN or the *rs*ODN for TIF2 are shown in **B** and **C**, respectively.

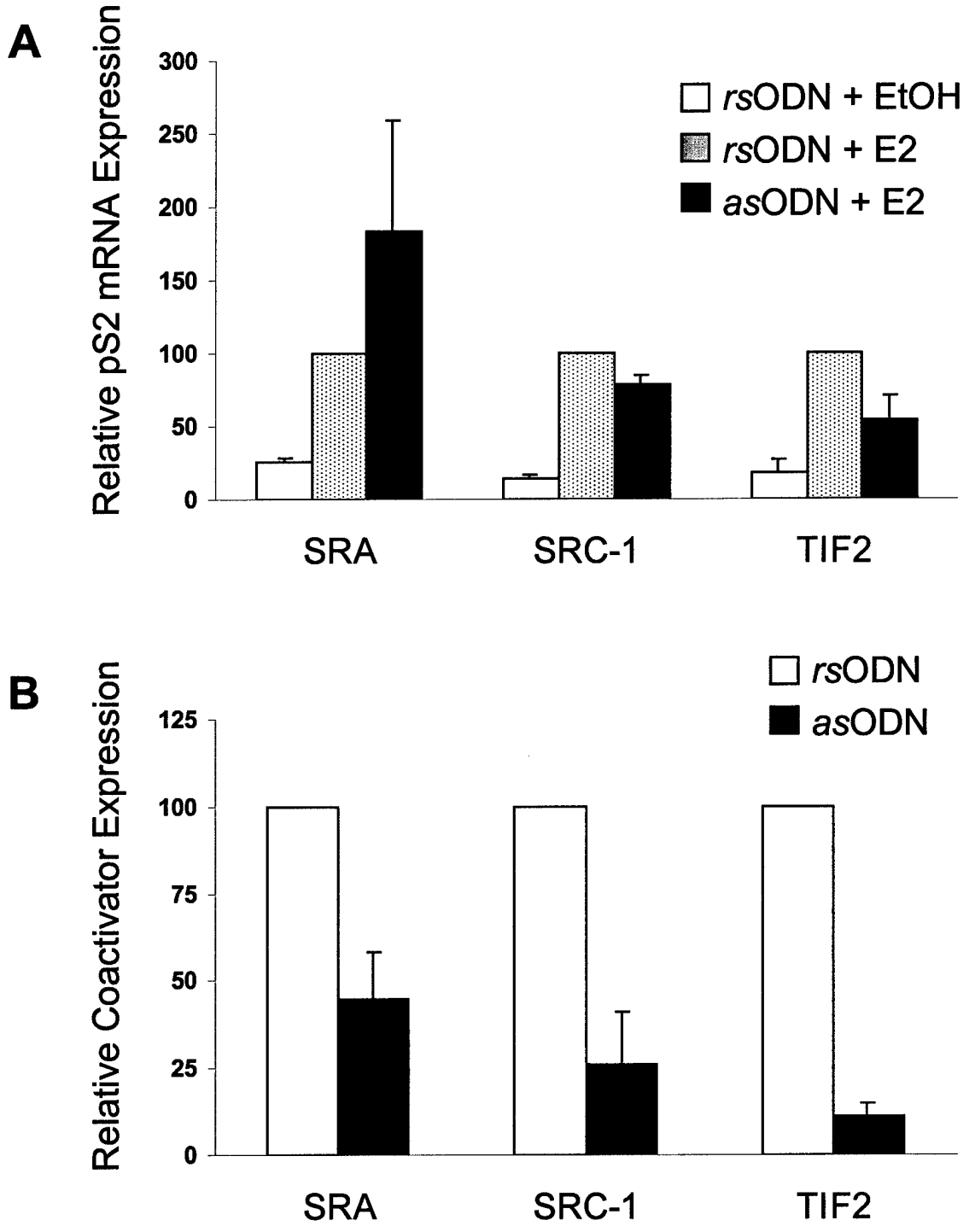


Figure 6: Inhibition of estrogen-induced pS2 mRNA expression by asODNs is coactivator-specific. **A)** MCF-7 cells grown in media containing 5% sFBS were transfected with 200 pmol of *rs*ODN or *as*ODN for the indicated coactivators and treated 24 h thereafter with 0.1% ethanol vehicle (EtOH) or 1 nM E2 for 16 h prior to harvesting cells for RNA isolation and quantitative pS2 and 18S RNA measurements by real-time RT-PCR. Values are presented relative to the pS2 mRNA levels normalized to 18S RNA values determined for estrogen and *rs*ODN treated samples (=100), and are given as the mean ± SEM for 3-5 independent experiments. **B)** Effect of *as*ODN on coactivator mRNA expression. MCF-7 cells were transfected with 200 pmol of *rs*ODN or *as*ODN for the indicated coactivator, and harvested 40 h later for RNA isolation and coactivator and 18S RNA measurements by real-time RT-PCR. Values are presented relative to the coactivator levels normalized for 18S levels determined for *rs*ODN treated samples (=100), and are given as the mean ± SEM for 3-6 independent experiments.

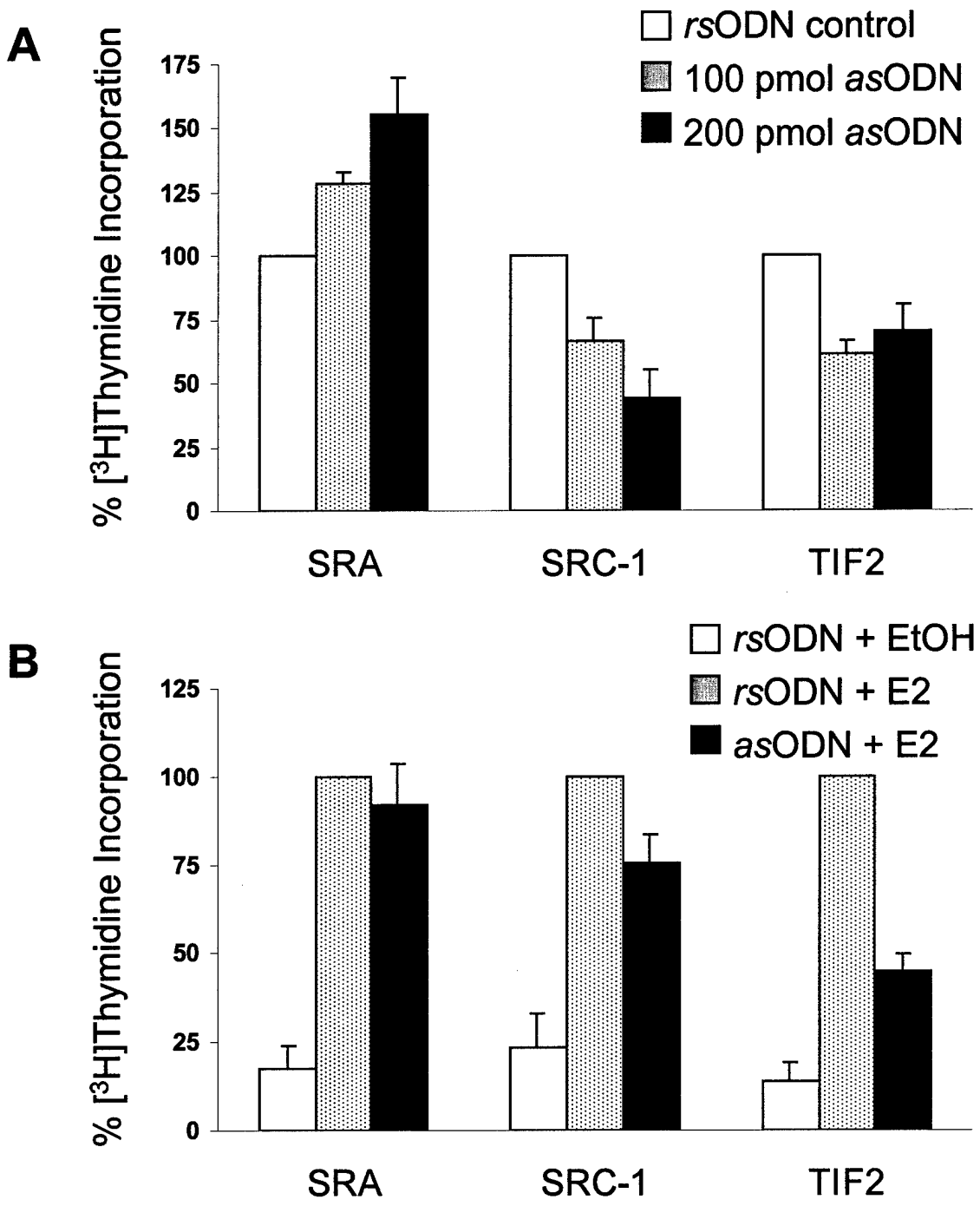


Figure 7: Inhibition of MCF-7 DNA synthesis by *as*ODNs is coactivator-specific. **(A)** Cells grown in media containing 10% FBS were treated with the indicated amounts of *as*ODNs and 24 hours after transfection, cell proliferation was assessed by [^{3}H]thymidine incorporation. Values are calculated as the percentage of incorporated counts in *as*ODN-treated cultures in comparison to the counts obtained in cultures transfected with the corresponding amount of *rs*ODN, and are given as the mean ± SEM for 3-4 independent experiments. **(B)** Cells grown in media containing 5% sFBS were transfected with 200 pmol of *rs*ODN or *as*ODN for the indicated coactivators and treated 24 h thereafter with ethanol vehicle (EtOH) or 1 nM E2 for 16 h to induce DNA synthesis. Values are calculated relative to the percentage of incorporated counts in *rs*ODN and E2 treated cultures (100%) for each coactivator, and are given as the mean ± SEM for 3-4 independent experiments.

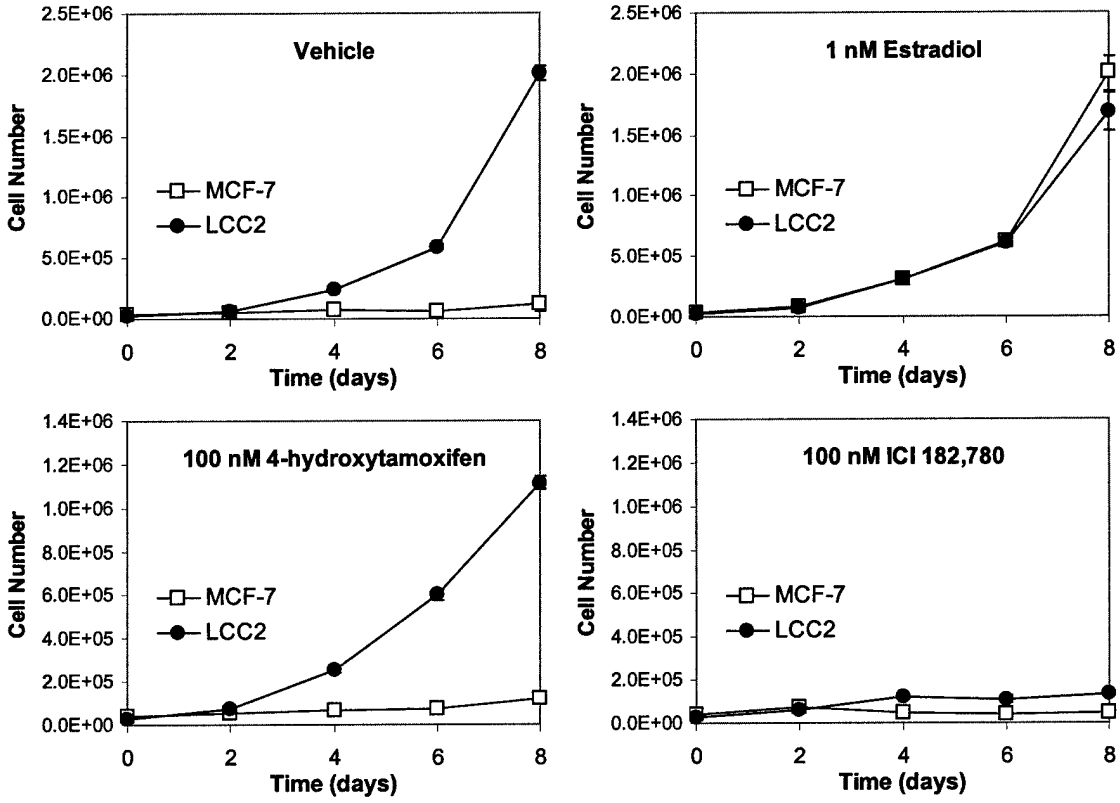


Figure 8: Effect of hormone treatment on growth of MCF-7 and LCC2 cells grown in phenol-red free DMEM & 10% sFBS.

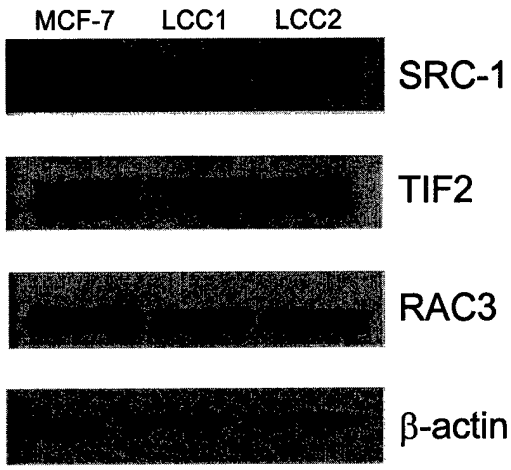


Figure 9: Western blot analysis of p160 coactivator expression in MCF-7, LCC1 and LCC2 cells.

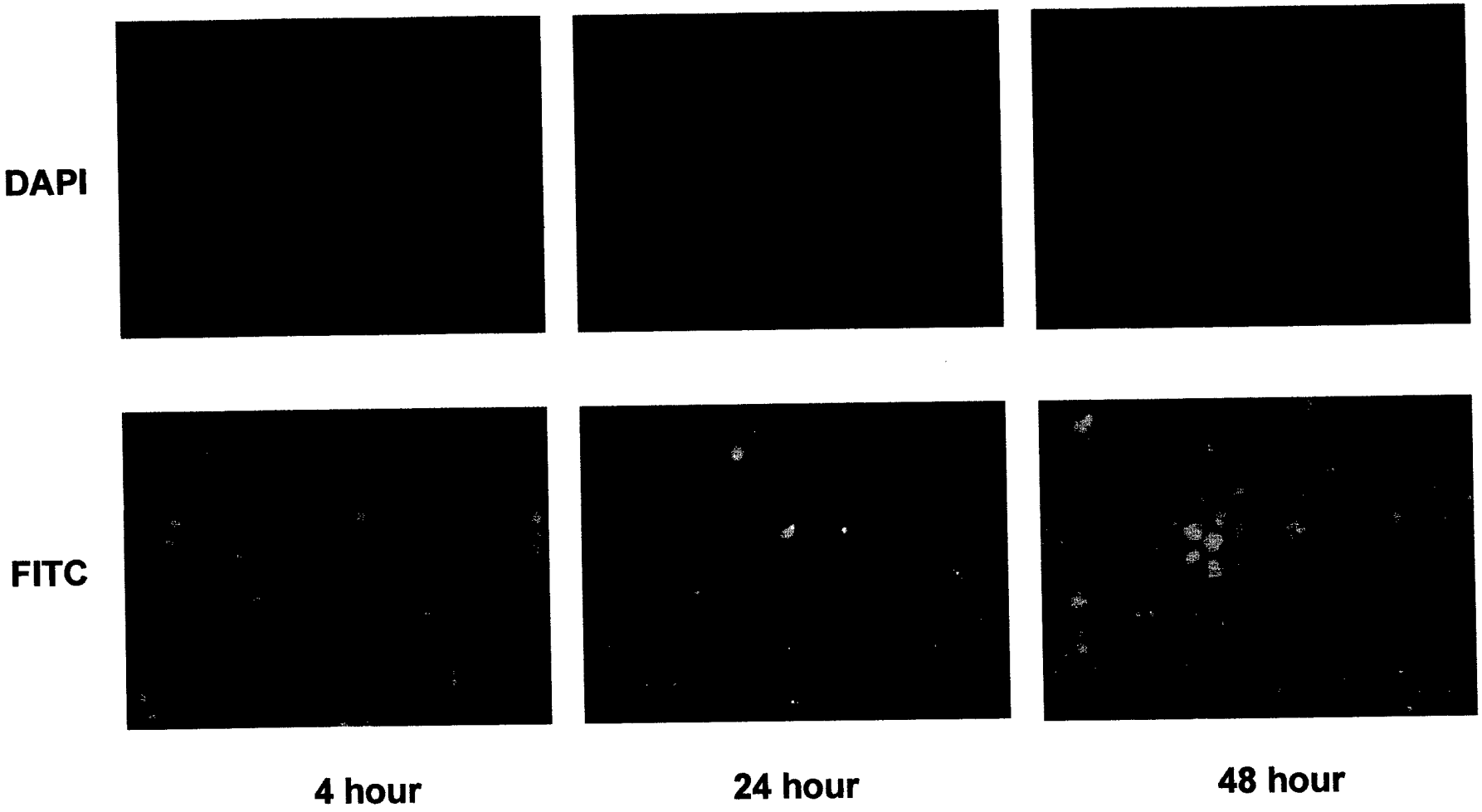


Figure 10. Uptake of a FITC-conjugated oligonucleotides by MCF-7 cells. Cells were transfected with a fluorescein-conjugated oligonucleotide and the efficiency of transfection was evaluated by observing microscopically the oligonucleotide fluorescence pattern (*bottom*) in comparison to the total number of cells observed by DAPI staining (*top*). Cells were processed for microscopy 4h (*left panels*), 24h (*middle panels*) or 48h (*right panels*) after removing the lipid/oligonucleotide mixture.

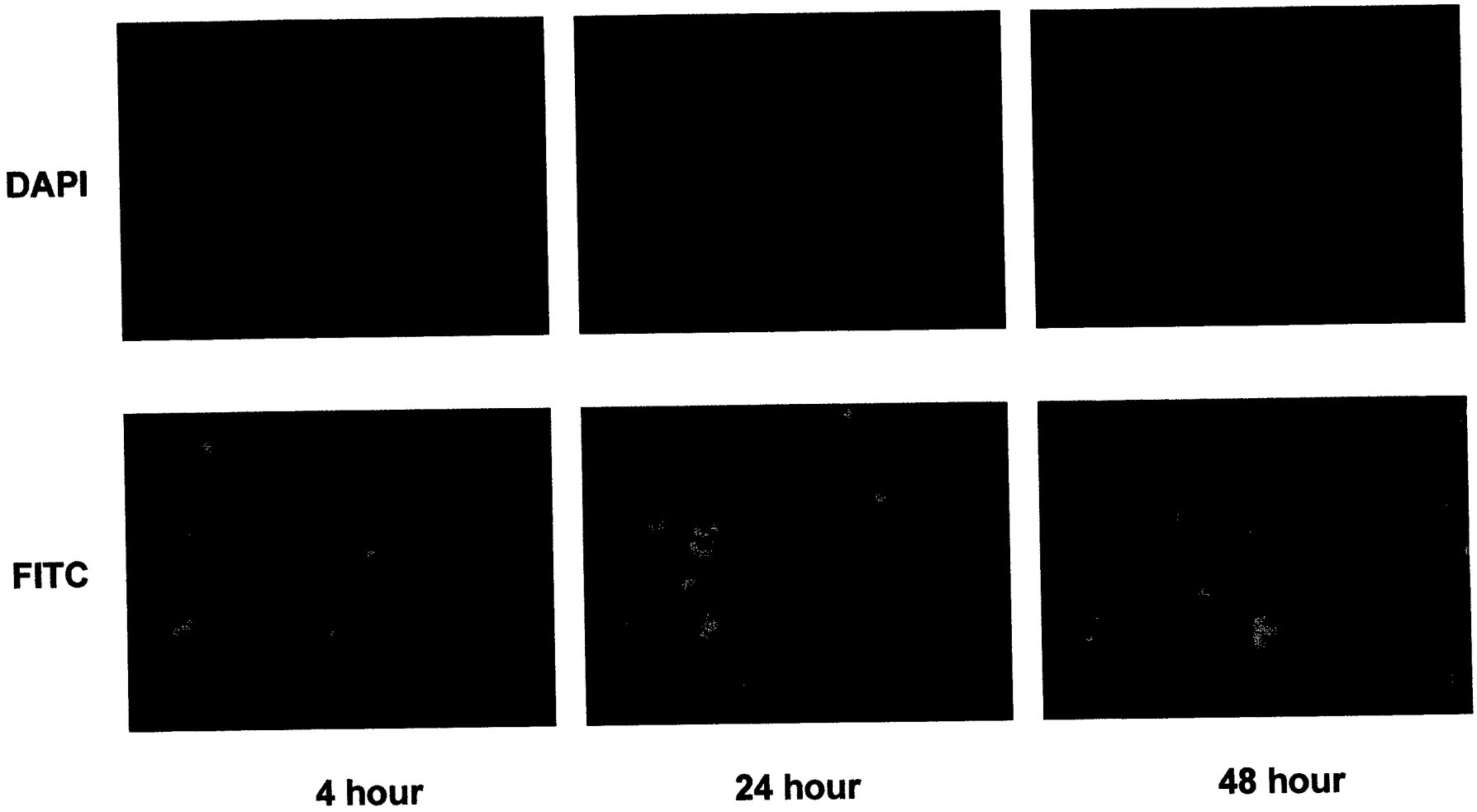


Figure 11. Uptake of a FITC-conjugated oligonucleotides by LCC1 cells. Cells were transfected with a fluorescein-conjugated oligonucleotide and the efficiency of transfection was evaluated by observing microscopically the oligonucleotide fluorescence pattern (*bottom*) in comparison to the total number of cells observed by DAPI staining (*top*). Cells were processed for microscopy 4h (*left panels*), 24h (*middle panels*) or 48h (*right panels*) after removing the lipid/oligonucleotide mixture.

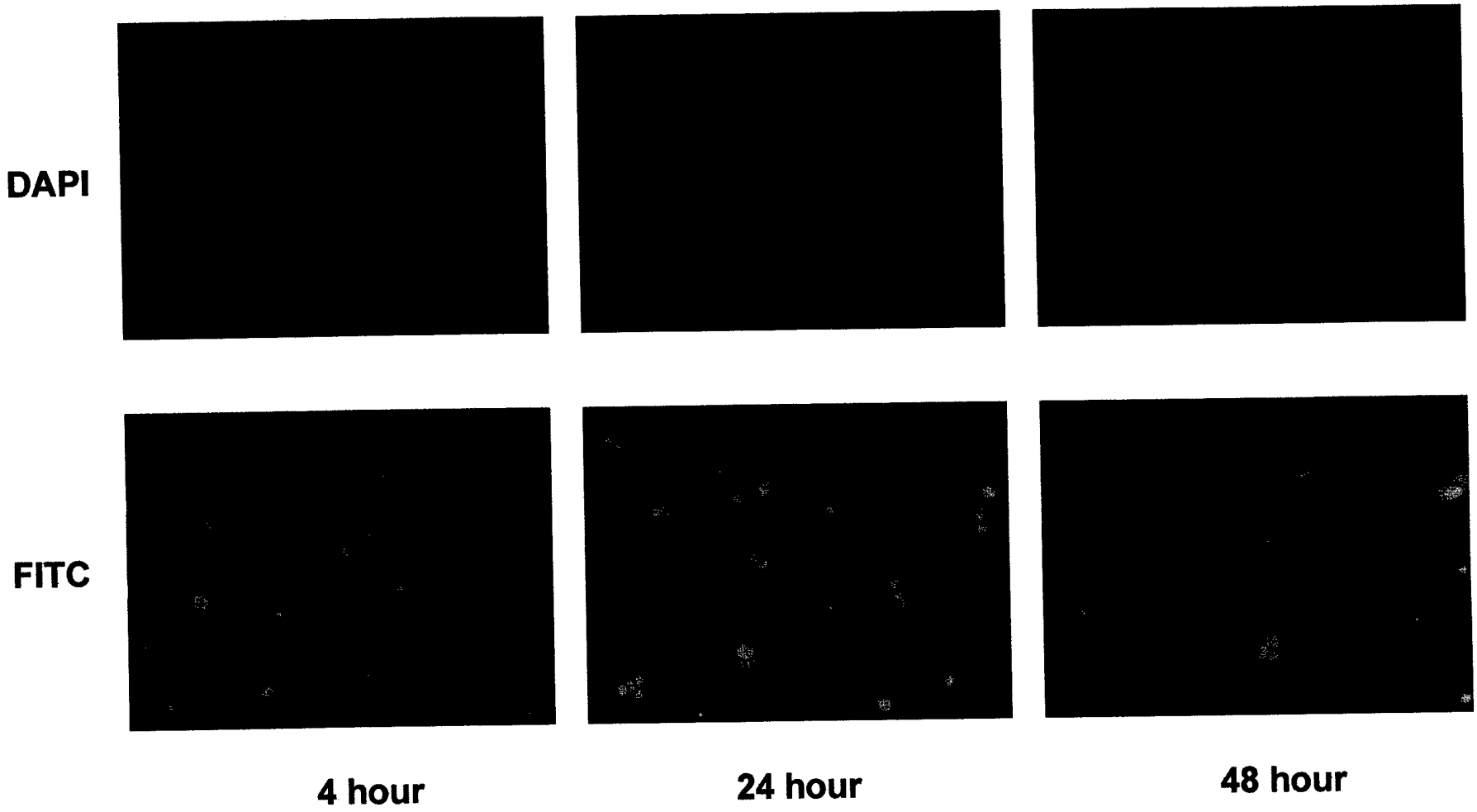


Figure 12. Uptake of a FITC-conjugated oligonucleotides by LCC2 cells. Cells were transfected with a fluorescein-conjugated oligonucleotide and the efficiency of transfection was evaluated by observing microscopically the oligonucleotide fluorescence pattern (*bottom*) in comparison to the total number of cells observed by DAPI staining (*top*). Cells were processed for microscopy 4h (*left panels*), 24h (*middle panels*) or 48h (*right panels*) after removing the lipid/oligonucleotide mixture.

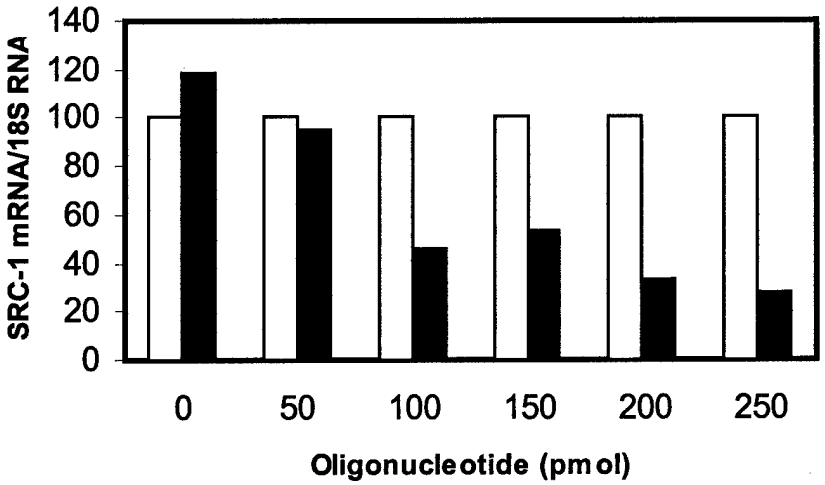


Figure 13: Inhibition of SRC-1 mRNA expression in MCF-7 cells treated with increasing amounts of random sense (■) or antisense (□) SRC-1 oligonucleotides. Total RNA was extracted and SRC-1 and 18S RNA levels were measured by real-time RT-PCR. Values are the average of two independent experiments.

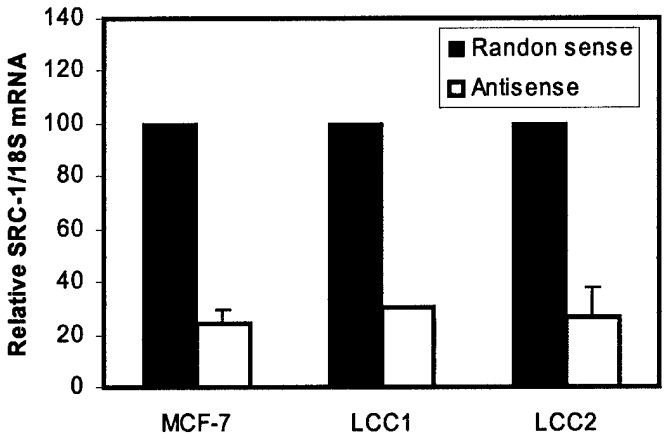


Figure 14: Inhibition of SRC-1 mRNA expression in cells treated with 200 pmol of random sense (■) or antisense (□) SRC-1 oligonucleotides. Total RNA was extracted and SRC-1 and 18S RNA levels were measured by real-time RT-PCR.

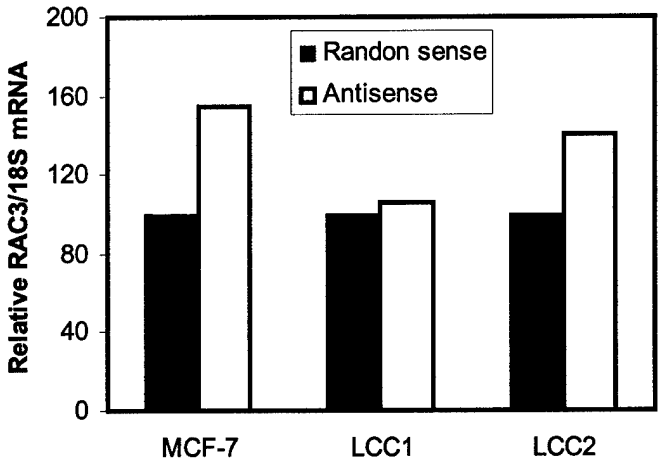


Figure 15: Inhibition of RAC3 mRNA expression in cells treated with 200 pmol random sense (■) or antisense (□) RAC3 oligonucleotides. Total RNA was extracted and RAC3 and 18S RNA levels were measured by real-time RT-PCR.

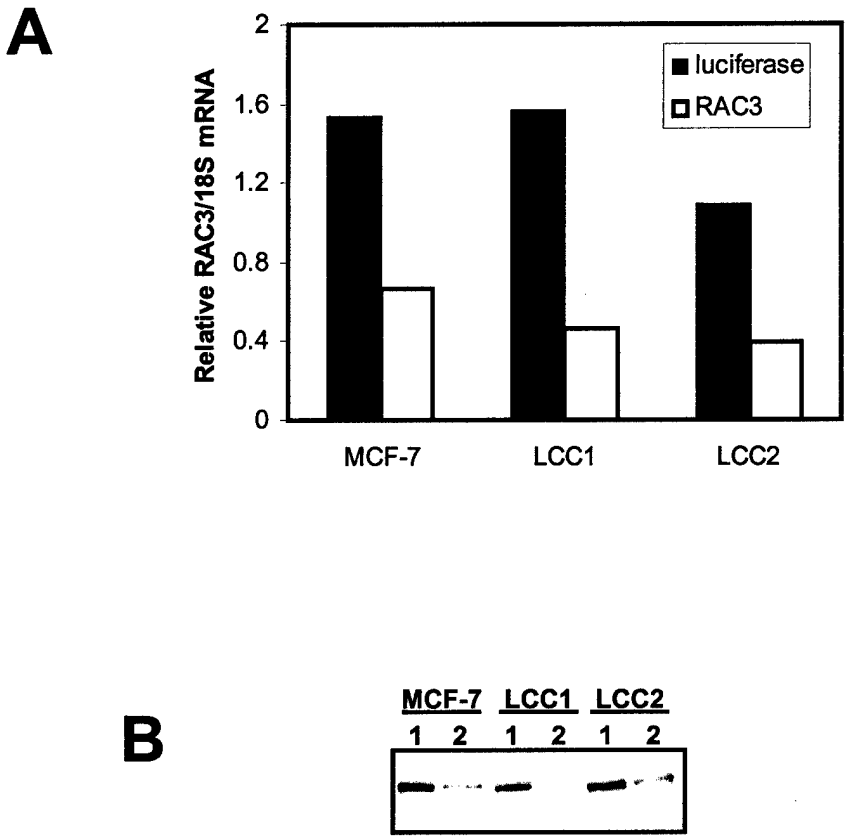


Figure 16: (A) Inhibition of RAC3 mRNA expression in cells treated with 100 pmol of siRNA against luciferase (■; a nonsense control) or RAC3 (□). Total RNA was extracted and RAC3 and 18S RNA levels were measured by real-time RT-PCR. (B) Western blot analysis of RAC3 protein expression in cells treated with either siRNA against luciferase (1) or RAC3 (2).

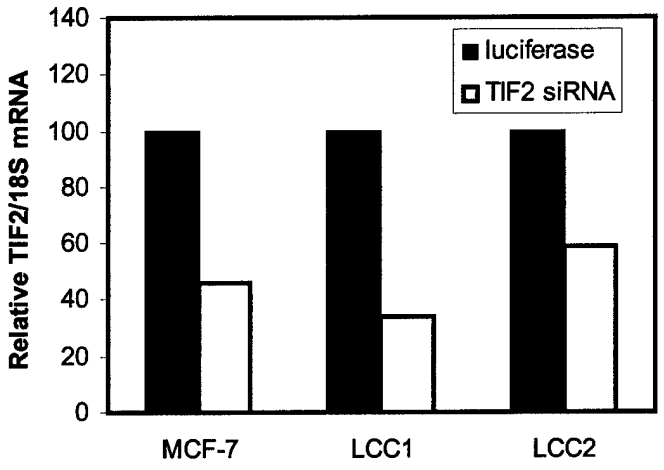
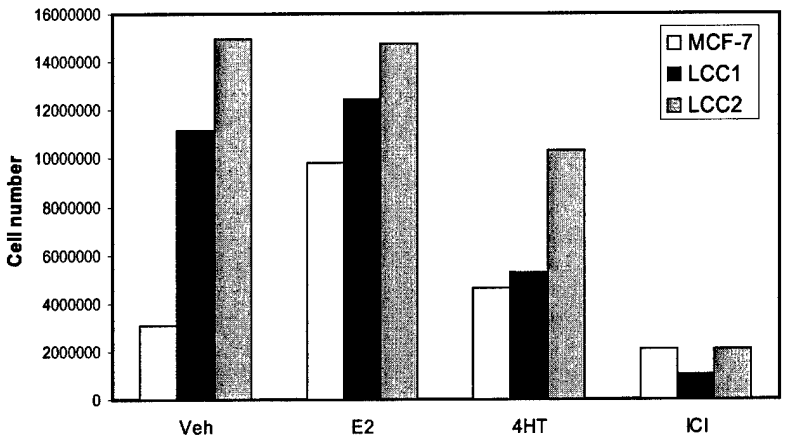


Figure 17: Inhibition of TIF2 mRNA expression in cells treated with 100 pmol of siRNA against luciferase (■) or TIF2 (□). Total RNA was extracted and TIF2 and 18S RNA levels were measured by real-time RT-PCR.

A



B

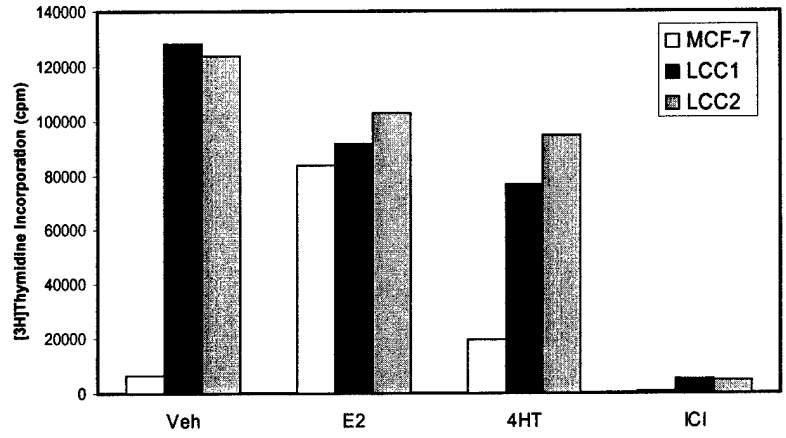


Figure 18: Comparison of methods for assessing the effect of 5 days of hormone treatment on cell growth. Panel A represents data reflecting cell number determined by Coulter counter. Panel B represents [^{3}H]thymidine incorporation in parallel cultures of cells.

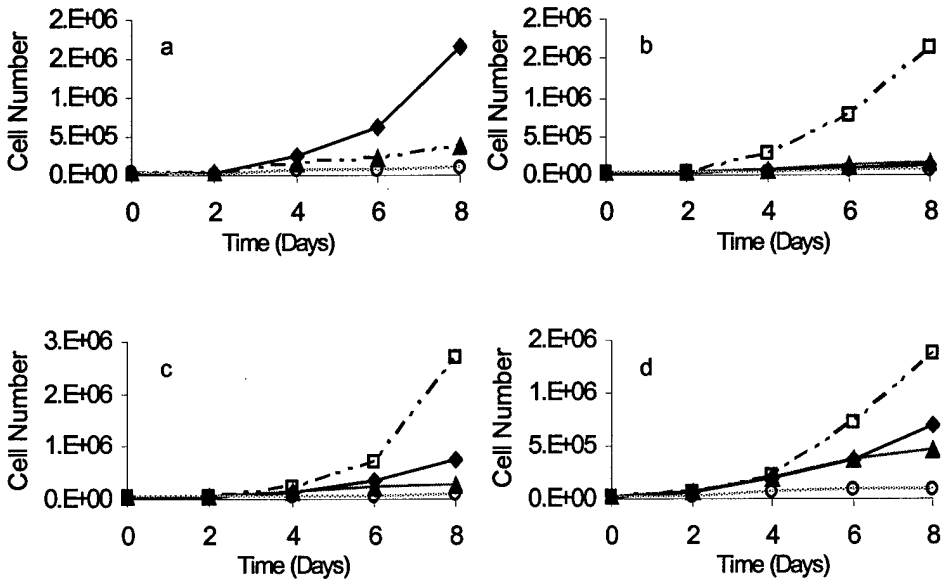


Figure 19: Cell number for a) MCF-7 (10% full serum), b) MCF-7 (10% stripped serum), c) LCC1 and d) LCC2 grown in the presence of ETOH (filled diamond), E2 (open square), 4HT (filled triangle), or ICI (open circle) for 8 days; cells were trypsinized and counted every 2 days.

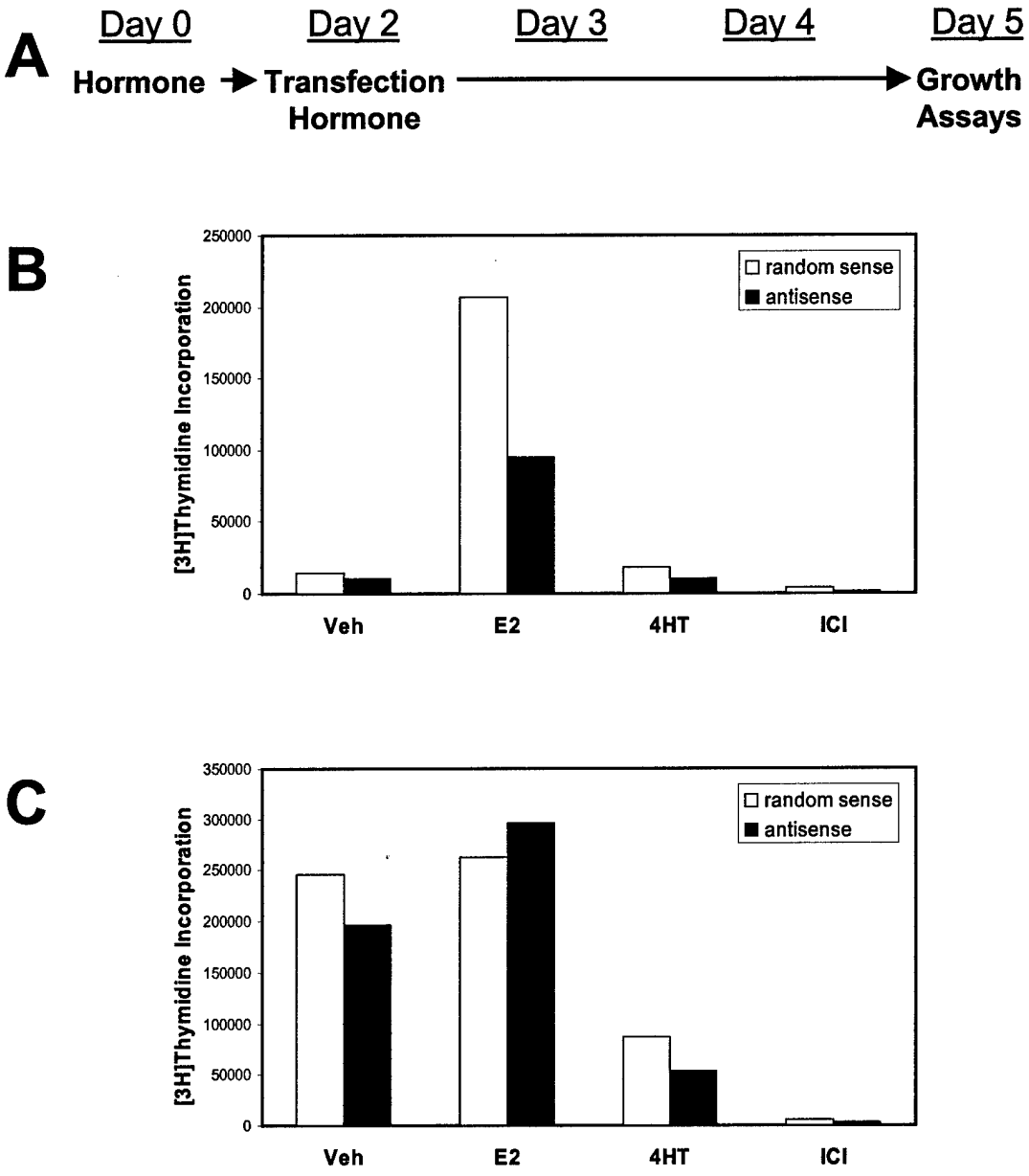


Figure 20: Inhibition of SRC-1 expression by antisense oligonucleotides inhibits estrogen-stimulated DNA synthesis in MCF-7 (*panel B*), but not LCC1 cells (*panel C*). The hormone treatment strategy is outlined in panel A. Cells were treated with either 0.1% ethanol (Veh), 1 nM E2, 100 nM 4HT or 100 nM ICI 182,780 for 2 days prior to transfection with either random sense or antisense oligonucleotides to SRC-1 mRNA. Following transfection, cells were fed with hormone-containing medium and the incubation continued for a further 3 days prior to assessing DNA synthesis by [^{3}H]thymidine incorporation assay.