

Dependence on Nuclear Localization Signals of the Opioid Growth Factor Receptor in the Regulation of Cell Proliferation

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The opioid growth factor receptor (OGFr) mediates the inhibitory action of OGF on cell replication of normal and neoplastic cells. The spatiotemporal course of OGFr nucleocytoplasmic trafficking was determined with a probe of full-length OGFr fused to enhanced green fluorescent protein (eGFP). Translation of OGFr required 8.5 hours, and transit into the nucleus required 8 hours; OGFr remained in the nucleus for 8 days. OGFr was initially expressed on the outer nuclear envelope, transited to the paranuclear cytoplasm, and into the nucleus. Transport through the nuclear pore was elucidated by mutation of the nuclear localization signal (NLS) sequences in full-length OGFr. Mutation of each NLS reduced nuclear localization by 5%–50%, whereas simultaneous mutation of NLS_{383–386} and NLS_{456–460} abolished OGFr-eGFP nuclear localization in 80% of the cells. To determine whether intact NLSs are important for the inhibition of cell proliferation, DNA synthesis was monitored with BrdU. Wild-type OGFr-eGFP-transfected cells had 20% BrdU-positive cells, whereas cells with simultaneous mutation of all three NLS sites had a 70% labeling index. These results indicate that the regulation of cell proliferation by the OGF-OGFr axis is dependent on nucleocytoplasmic translocation and reliant on the integrity of two NLSs in OGFr to interact with transport receptors. *Exp Biol Med* 234:532–541, 2009

Key words: nucleocytoplasmic transport; nuclear localization signal; cell proliferation; opioid growth factor receptor; opioid growth factor; green fluorescent protein

Introduction

The opioid growth factor (OGF), chemically termed [Met⁵]-enkephalin, is a pentapeptide that is constitutively expressed, autocrine produced, and secreted to inhibit the proliferation of normal and neoplastic cells (1). The action of OGF is tonic, stereospecific, reversible, non-cytotoxic and non-apoptotic inducing, not associated with differentiative, migratory, invasive, or adhesive processes, independent of serum, anchorage-independent, and occurs at physiologically relevant concentrations (1). OGF is targeted to DNA synthesis (2–5), directed toward the G₁-S interface of the cell cycle (6), and focused on upregulation of cyclin-dependent kinase inhibitory pathways (7–9).

The action of OGF is mediated by interaction with the OGFr receptor (OGFr). The gene for human OGFr is composed of seven exons and six introns, and encodes a 677 amino acid protein (1). OGFr has an apparent mass of 62 kDa. The chromosomal location of the human OGFr is 20q13.3 (1). Although OGFr has pharmacological/biochemical characteristics of a classical opioid receptor (recognizes opioids, naloxone reversibility, stereospecificity), there is no homology of OGFr with classical opioid receptors at the nucleotide or amino acid levels (1). Antisense experiments with OGFr, as well as continuous blockade of OGFr with the general opioid antagonist naltrexone (NTX), support the regulatory role of the OGF-OGFr axis as a tonically active inhibitory system targeted to cell replication and homeostasis, and that is ligand-dependent for function (1).

Previous immunohistochemistry and immunoelectron microscopic studies (10, 11) exploring nucleocytoplasmic trafficking of the OGF-OGFr axis have documented that OGF and OGFr are colocalized in both the cytoplasm and the nucleus. OGF alone is found in the cytoplasm, and OGFr alone can be recorded on the outer nuclear envelope. OGF and OGFr are colocalized on the outer nuclear envelope, in the paranuclear cytoplasm, perpendicular to the nuclear envelope in the putative nuclear pore complex,

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and in the nucleus adjacent to heterochromatin. Analysis of the nucleotide structure of OGFr reveals 3 nuclear localization signal (NLS) sequence: one bipartite and two monopartite. NLSs are typically short stretches of basic amino acid-rich sequences (12, 13) that interact with karyopherin (importin) β , either directly or through the adapter karyopherin α , to translocate a cargo through the nuclear pore complex (14–17). Using immunoelectron microscopy, karyopherin β has been colocalized to the OGF-OGFr complex in the cytoplasm and the nucleus (10, 11), suggesting that karyopherin β may be functioning as a nucleocytoplasmic transport receptor. These previous studies raised questions about the dynamics of the OGF-OGFr and the dependence on one or more NLS sequences for transport from the cytoplasm to the nucleus.

The present investigation examined the spatiotemporal relationship of OGFr in human cells by using a fusion protein of OGFr tagged with eGFP (i.e., OGFr-eGFP) and analysis using deconvolution microscopy. To gain molecular insight into the mechanism of OGFr nucleocytoplasmic trafficking, the repercussions of mutation of each NLS site, as well as combinations of these mutated NLS sites, were evaluated. Finally, to ascertain the functional implications of the mutation of these NLS sites, BrdU was utilized to evaluate DNA synthesis. The data reveal that (i) OGFr undergoes a specific course of trafficking between the cytoplasm and the nucleus, (ii) specific NLS sequences must be intact for the translocation of OGFr across the nuclear envelope that is critical for regulation of cell proliferation, (iii) OGFr must be imported from the cytoplasm to the nucleus in order to modulate DNA synthesis, and (iv) OGFr does not undergo nuclear export.

Materials and Methods

Cell Culture. The human head and neck squamous cancer cell line SCC-1, obtained from the University of Michigan Cancer Research Laboratory (Thomas E. Carey, Ph.D., Director), was utilized in these studies because of its high transfection efficiency (18). The cells were cultured in DMEM (The Pennsylvania State University). Human pancreatic cancer cell line, BxPC-3, was obtained from the American Type Culture Collection (Manassa, VA) and cultured in RPMI (The Pennsylvania State University). All media were supplemented with 10% fetal bovine serum, 1.2% sodium bicarbonate, and 5000 units/ml penicillin, 5 μ g/ml streptomycin, and 10 μ g/ml neomycin. Cells were grown at 37°C in a 5% CO₂/95% air-humidified incubator.

Plasmid Constructs. The OGFr constructs were generated by PCR amplification of the human full-length isoform. These cDNA fragments were then subcloned into the pEGFP N1 vector. The PCR primers used in each reaction incorporated EcoR1 and Sal1 restriction sites into the PCR products at the 5' and 3' ends, respectively. The primers used to perform the PCR amplifications were: Zag152 5'-GAATTC TAGCCGAGCATGGACGACC-3'

and Zag153 5'-GTCGACTTCCAGACTTGG CAGAA-GACT-3'; restriction sites are underlined. The PCR products were ethanol precipitated, digested, and ligated into the pEGFP N1 vector.

OGFr Possesses Three Putative NLS Sites. Inspection of the OGFr amino acid sequence with WOLF PSORT software revealed three basic regions (NLS_{267–296}, NLS_{383–386}, NLS_{456–460}) that are highly conserved in diverse species, with the potential to serve as NLS sites. To determine the relevance of the basic regions as putative NLS sites in the context of the full-length OGFr, we created a panel of OGFr point mutant constructs (Fig. 1). Mutants A1, A2, and A3 had mutations within NLS_{267–296}. Mutant B had mutations within NLS_{383–386}. Mutant C had mutations within NLS_{456–460}. Mutant D had mutations within NLS_{267–296} and NLS_{383–386}. Mutant E had mutations within NLS_{383–386} and NLS_{456–460}. Mutant F had mutations within NLS_{267–296}, NLS_{383–386}, and NLS_{456–460}. Mutant G had mutations within NLS_{267–296} and NLS_{456–460}. Each of these mutants was tested for nucleocytoplasmic transport and signaling of cell proliferation.

Site-Directed Mutagenesis. The primers and templates for the putative NLS mutants are provided in Table 1. Mutagenesis reactions were performed using the Quik-change Site-Directed Mutagenesis Kit (Stratagene, Agilent Technologies, Santa Clara, CA) according to the manufacturer's protocol. In each PCR reaction, 50 ng of template DNA was used. All expression constructs were verified by sequencing.

The primers employed incorporated the mutations and were as follows: NLS_{267–296}: Zag197, 5'-GCCGTGCGCTGTCGACACCAGGCCGCCAGCTGGTGC-3'; Zag198, 5'-GCACCAGCTGGG-CGGCCTGGTGTCGACAGCGCACGGC-3'; Zag215, 5'-CTGGGAGCACTTCGCGCCCGCCTGCGCGTT-CGTCTGGGGG-3'; Zag216, 5'-CCCCCAGACGAA-CGCGCAGGCGGGCGCGAAGTGCTCCCAG-3'; Zag217, 5'-CCCCAAGACAAGCTGGCGGCG-TTCGTGCCAGCTCTCTGCC-3'; Zag218, 5'-GG-CAGAGAGCTGGGCGCGAACGCCGCCACGTT-GTCTTG GGG-3'. NLS_{383–386}: Zag225, 5'-TTAAGCCCCAAAGAGAGCGCGGCGAGGAA-GCTGGAGCTGAG-3'; Zag226, 5'-TCAGCTCCAGC-TTCCTCGCCGCGCTCTCTTTGGGGCTTAA-3'. NLS_{456–460}: Zag227, 5'-TGGCCGACAAGGT-GAGGGCGGCGAGGAAGGTGGATGAGG-3'; Zag228, 5'-CCTCATCCACCTTCCT CGCCGCCCTCACC-TTGTCGGCCA-3'.

The Zag197 and Zag198 primers with incorporated Sal 1 restriction sites (GCCGAC→GTCGAC) of the mutants did not alter the amino acid result.

Transient Transfections. Prior to transfection, SCC-1 cells were seeded onto coverslips in 24-well dishes or 35-mm glass bottom culture dishes (MatTek, Ashland, MA) in antibiotic-free DMEM media. The cells were incubated for at least 12 h until cultures were 50%–70%

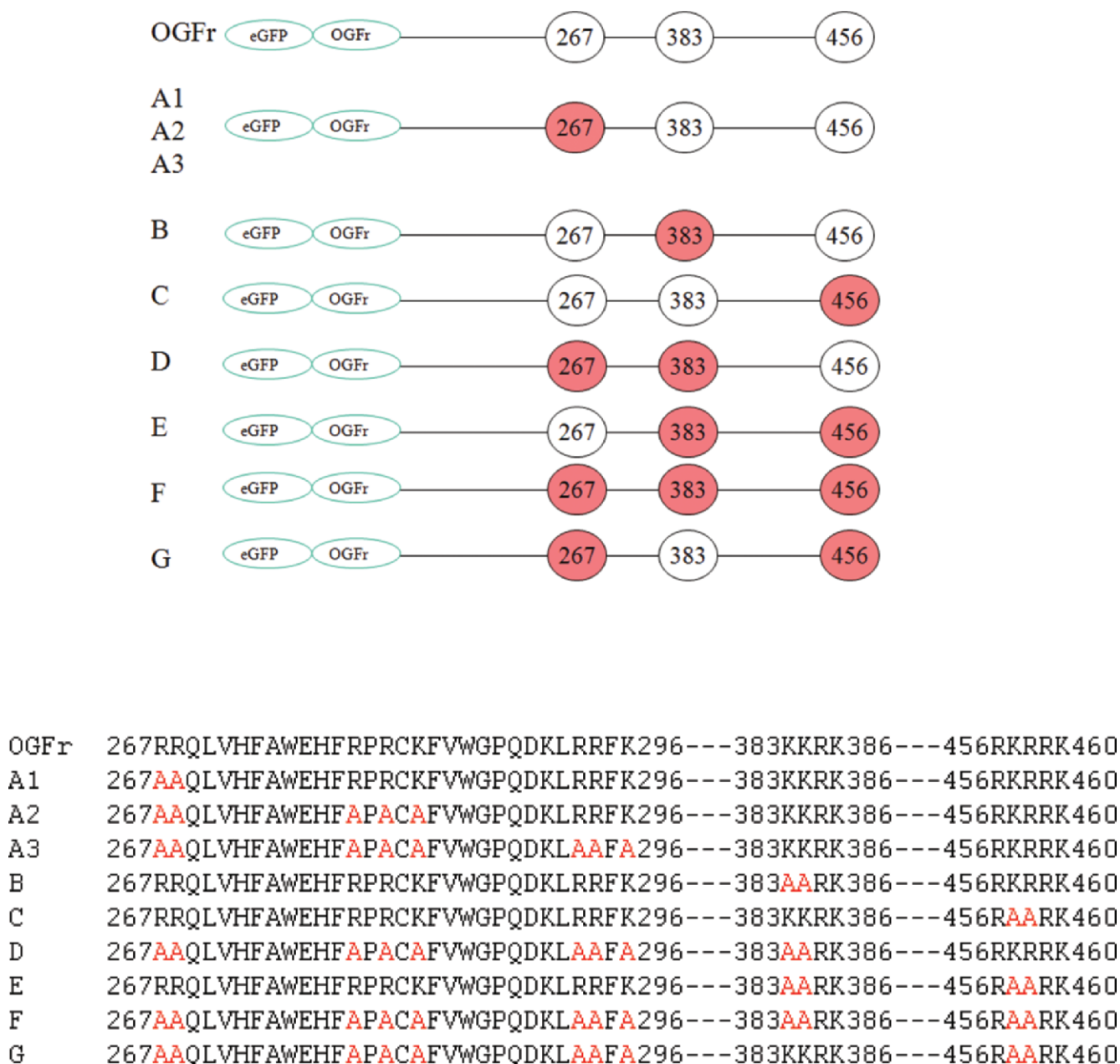


Figure 1. Schematic representation of the OGFr-eGFP mutants used to identify the NLSs. Inspection of the OGFr amino acid sequence with WOLF PSORT software revealed three basic regions (NLS₂₆₇₋₂₉₆, NLS₃₈₃₋₃₈₆, NLS₄₅₆₋₄₆₀) that are highly conserved in diverse species, with the potential to serve as NLS sites. Mutants A1, A2, and A3 had mutations within NLS₂₆₇₋₂₉₆; Mutant B had mutations within NLS₃₈₃₋₃₈₆; Mutant C had mutations within NLS₄₅₆₋₄₆₀; Mutant D had mutations within NLS₂₆₇₋₂₉₆ and NLS₃₈₃₋₃₈₆; Mutant E had mutations within NLS₃₈₃₋₃₈₆ and NLS₄₅₆₋₄₆₀; Mutant F had mutations within NLS₂₆₇₋₂₉₆, NLS₃₈₃₋₃₈₆, and NLS₄₅₆₋₄₆₀; and Mutant G had mutations within NLS₂₆₇₋₂₉₆ and NLS₄₅₆₋₄₆₀. A color version of this figure is available in the online journal.

confluent. For each construct, 1 μ g of plasmid DNA and 1 μ l Lipofectamine 2000 (Invitrogen, Carlsbad, CA) were diluted in 50 μ l serum-free, antibiotic-free DMEM media. In 35-mm glass bottom culture dishes, for each construct, 5 μ g of plasmid DNA and 5 μ l Lipofectamine 2000 were diluted in 250 μ l serum-free, antibiotic-free DMEM media. These mixtures were incubated at 20°C for 15 min to allow reagent/DNA complex formation. The complex was added to the cells in fresh, serum-free, antibiotic-free DMEM

media, followed by incubation for 4 h at 37°C, after which the reagent was aspirated and the cells were incubated in serum-supplemented DMEM medium.

Immunofluorescence Microscopy. The subcellular localization of wild-type OGFr-eGFP was analyzed by fluorescent microscopy with deconvolution optics (Olympus IX81, Center Valley, PA) every 30 mins for the first 9 h, and every day thereafter for 9 days after initiation of transfection in living SCC-1 cells. In brief, the cells were stained with

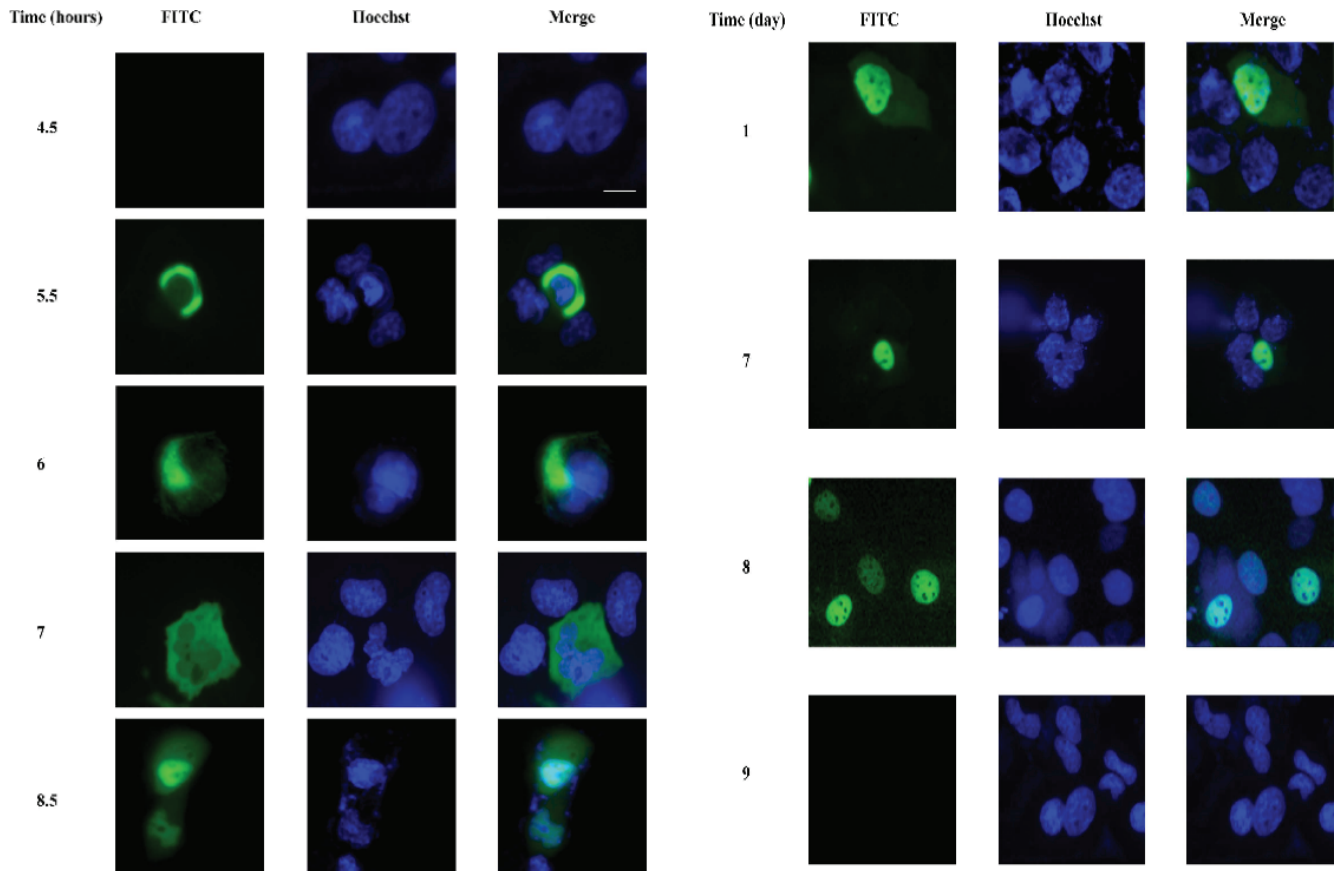


Figure 2. Time course of subcellular localization and expression of OGFr-eGFP. Live SCC1 cells were transfected with OGFr-eGFP for 4 h. Deconvolution microscopy was utilized to visualize the structural relationships from 4 to 9 h (Fig. 1A) and at 24-h intervals for 9 days (Fig. 1B). Photomicrographs represent the projection image of all Z-stack images. At 5.5 h from the initiation of transfection, OGFr-eGFP was detected proximal to the periphery of the nucleus, indicating the time needed to translate the OGFr protein. At 7 h following the initiation of transfection, OGFr-eGFP was found in the cytoplasm, and at 8.5 h, OGFr-eGFP was observed in the cytoplasm and cell nucleus. At 24 h after initiation of transfection, OGFr-eGFP was localized only in the nucleus, and remained in that location for up to 8 days. Bar = 5 μ m. A color version of this figure is available in the online journal.

Hoechst's for 5 min in order to visualize nuclei, and were washed twice with media prior to observation.

The BrdU incorporation and the subcellular localization of wild-type OGFr-eGFP and NLS mutants were analyzed by immunostaining. SCC-1 cells growing 22-mm² coverslips were transfected for 4 h with different constructs and incubated in serum-supplemented DMEM medium for an additional 20 h. At that time, fresh complete DMEM

medium with 30 μ M BrdU (Sigma) was added to each culture for 3 h. The cells were washed twice with 1X PBS before and after fixing with fresh 4% paraformaldehyde for 10 min. The cells were treated with 2 N HCl and blocked with 4% fetal calf serum in PBS for 30 min. After blocking, mouse Alexa-594-conjugated anti-BrdU antibody (Invitrogen) and rabbit Alexa-488-conjugated anti-GFP antibody (Invitrogen) were added at 1:200 dilution in 1% normal goat serum-PBS and incubated for 45 min at 37°C. Following a wash with PBS, the cells were stained with DAPI (1 μ g/ml) and sealed onto glass slides. At least 150 cells/group with at least 50 cells/experiment $\times$ 3 experiments were counted in a masked fashion.

Statistical Analysis. Data for BrdU incorporation were evaluated using the two-tailed Student's *t* test.

Results

Spatial and Temporal Course of OGFr-eGFP.

OGFr-eGFP was first visible 5.5 h after the initiation of transfection, indicating the time-frame for translation of OGFr (Fig. 2A, B). At 5.5 h, OGFr-eGFP was detected

Table 1. Primers Utilized to Design Mutant Plasmids

Mutants	Template	Primers
A1	OGFr-eGFP	Zag197, Zag198
A2	A1	Zag215, Zag216
A3	A2	Zag217, Zag218
B	OGFr-eGFP	Zag225, Zag226
C	OGFr-eGFP	Zag227, Zag228
D	A3	Zag225, Zag226
E	B	Zag227, Zag228
F	D	Zag227, Zag228
G	A3	Zag227, Zag228

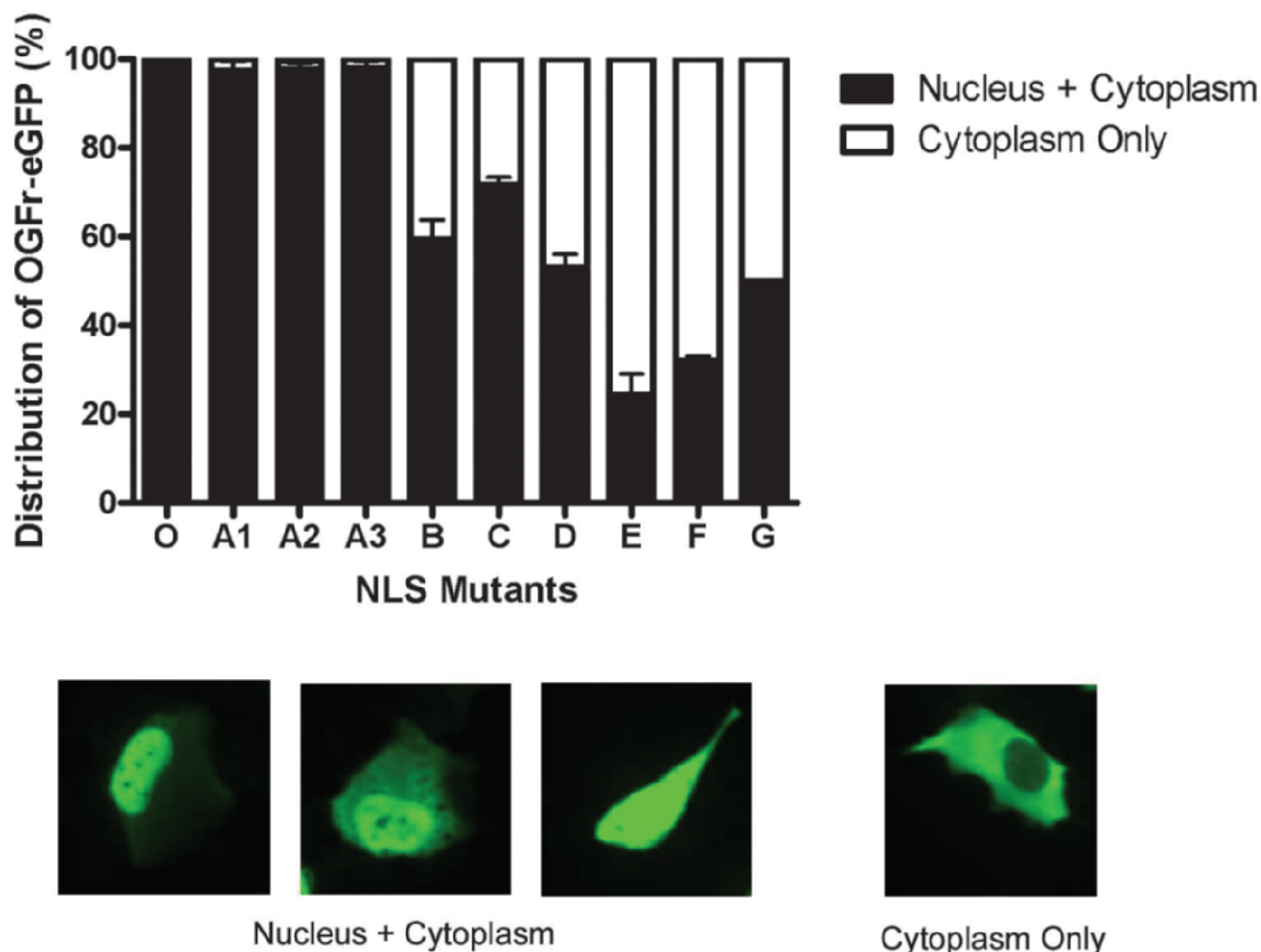


Figure 3. The subcellular distribution of nonmutated OGFr-eGFP (O) and NLS-mutated OGFr-eGFP after 24 h of transfection. At least 150 cells were scored for OGFr-eGFP localization in each of three independent experiments. Cells were classified by fluorescent staining in the nucleus and cytoplasm or cytoplasm only. The data represent means $\pm$ SEM for the percentage of distribution of OGFr-eGFP. A color version of this figure is available in the online journal.

proximal to the periphery of the nucleus, and appeared to be associated with the nuclear envelope. Seven hours following transfection, OGFr-eGFP was found in the cytoplasm and, at 8.5 h after transfection, OGFr-eGFP was observed in the cytoplasm, as well as in the cell nucleus. At the 24-h time point, OGFr-eGFP was localized only in the nucleus, and it remained in that location for up to 8 days after initiation of transfection. On Day 9, OGFr-eGFP was not detected anywhere in the SCC-1 cells. Similar results were observed in human pancreatic cancer BxPC-3 cells (data not shown).

NLS-Regulated Nuclear Localization of OGFr.

To determine the relevance of the basic regions of OGFr as putative NLS sites, we transfected wild-type OGFr-eGFP or mutant OGFr-eGFP constructs into SCC-1 cells (Fig. 3). At 24 h after initiation of transfection, OGFr-eGFP was detected both in the cytoplasm and the nucleus of 100% of control SCC-1 cells. Cells transfected with mutations A1, A2, and A3 at NLS_{267–296} had a similar distribution of eGFP as the

control SCC-1 cells. For mutants B and C, which had mutations in NLS_{383–386} and NLS_{456–460}, respectively, 30%–40% of the eGFP was localized only to the cytoplasm. For mutant D, with mutations in NLS_{267–296} and NLS_{383–386}, 45% of the OGFr-eGFP was localized only to the cytoplasm. When NLS_{383–386} and NLS_{456–460} were mutated (mutant E), 80% of the OGFr-eGFP was distributed only to the cytoplasm. When all three NLS sites were mutated (mutant F), 70% of the OGFr-eGFP was detected only in the cytoplasm. Finally, when mutations in NLS_{267–296} and NLS_{456–460} were observed with OGFr-eGFP, approximately 50% of the fluorescence signal was only localized in the cytoplasm. Thus, we may conclude that (1) NLS_{267–296} does not play a role in regulating OGFr nuclear import and (2) an intact NLS_{383–386} and NLS_{456–460} are required for efficient OGFr nuclear localization.

The Functional Importance of the NLS Sites in OGFr to Inhibit Cell Proliferation. To determine

whether the NLS sites of OGFr are important to the function of the OGF-OGFr axis as an inhibitory pathway of cell proliferation, we examined DNA synthesis after transfection with NLS-mutated OGFr-eGFP (Figs. 4, 5). While the deconvolution microscopic images shown in Fig. 4 are representative of the population of cells expressing each of the constructs along with BrdU incorporation, there was some heterogeneity in the distribution of fluorescence. To quantitate these differences, at least 150 individual cells from three experiments that were not transfected, or those transfected with eGFP (control for transfection), OGFr-eGFP (control for OGFr overexpression) or mutant F (NLS₂₆₇₋₂₉₆, NLS₃₈₃₋₃₈₆, and NLS₄₅₆₋₄₆₀), were scored for BrdU incorporation. Approximately 50% (range of 47%–55% depending on the experiment performed) of the cells were BrdU positive in untransfected cultures, and the group transfected with eGFP only had 51% of the cells that incorporated BrdU. Cells transfected with OGFr-eGFP had only 22% of the cells that were BrdU positive, and this was a significant decrease from the untransfected group included in the experiment. Finally, cells transfected with mutant F had 68% of the cells incorporating BrdU, and this was a significant increase from the untransfected group.

Discussion

This study shows for the first time the spatial and temporal parameters of OGFr trafficking between the cytoplasmic and nuclear compartments in human cells, and has resolved issues of subcellular localization and receptor dynamics in living cells (Fig. 6A). Using an OGFr-eGFP probe, OGFr was initially located on the periphery of the nuclear envelope, rapidly transited to the paranuclear cytoplasm, and entered the nucleus shortly thereafter. Taken together with previous results using immunoelectron microscopy with immunogold techniques and antibodies specific for OGF and OGFr, our previous hypothetical model of the putative intracellular trafficking pattern of OGFr in normal and neoplastic cells can be elucidated. First, OGFr protein requires at least 5.5 h for translation. Second, the OGF receptor first appears on the nuclear envelope, with immunoelectron microscopy showing that OGFr is specifically assembled proximal to the outer nuclear envelope. Third, within the next 1.5 h, OGFr interacts with OGF at the outer nuclear envelope, thereby indicating that the receptor initially interfaces with the peptide at the site of receptor translation. Fourth, OGFr (along with OGF) is situated in the paranuclear cytoplasm for another 1.5 h, suggesting that the OGF-OGFr complex detaches from the outer nuclear envelope. Fifth, 8.5 h after transfection, OGFr can be detected in the nucleus, documenting that the OGF-OGFr complex traffics from the cytoplasmic compartment to the nuclear compartment. Sixth, OGFr can be observed solely in the nucleus after 24 h, inferring that nuclear export does not occur and that the OGF-OGFr pathway is unidirectional from the cytoplasm to the nucleus. Seventh, the absence of

nuclear export using OGFr-eGFP reveals that the OGF receptor (and OGF) does not undergo recycling, indicating that a determinant of OGF-OGFr action relies on the synthesis rather than the reuse of OGFr. These data also concur with nucleotide analysis of OGFr documenting no nuclear export signals. Eighth, OGFr remains in the nucleus for up to 8 days, possibly indicating either an extended time for OGF-OGFr functional activity and/or slow degradation of the OGF-OGFr complex.

The present investigation also explains that the robust nuclear localization of the OGF-OGFr complex (10, 11) is due to mechanisms involving nuclear localization sequences encoded by OGFr (Fig. 6A). Of the three NLS sites, the two monopartite sequences (NLS₃₈₃₋₃₈₆, NLS₄₅₆₋₄₆₀), but not the singular bipartite sequence (NLS₂₆₇₋₂₉₆), were discovered to be essential to shuttling of OGF-OGFr through the nuclear pore complex. Thus, mutation of either NLS₃₈₃₋₃₈₆ or NLS₄₅₆₋₄₆₀ restricted passage of OGFr-eGFP from the cytoplasm to the nucleus by up to 50%, whereas mutation of NLS₂₆₇₋₂₉₆ only limited OGFr-eGFP by 5%. The full magnitude of the requisite nature of specific NLSs to nucleocytoplasmic transport was documented in combinatorial experiments with the NLS mutants. Up to 80% of OGFr-eGFP nuclear localization was abolished when both NLS₃₈₃₋₃₈₆ and NLS₄₅₆₋₄₆₀ were simultaneously mutated, and this effect was no greater than when all three NLS sites were mutated. Whether the remaining 20% of the OGFr-eGFP that is in both the nucleus and cytoplasm reflects an artifact in the methodology used or if other pathways are involved remains to be investigated. Therefore, our study makes the novel finding that signal-mediated transport related to two NLS sites (NLS₃₈₃₋₃₈₆ and NLS₄₅₆₋₄₆₀) encoded in OGFr is imperative to the translocation of this receptor (and its peptide) from the cytoplasm to the nucleus.

Given the nucleocytoplasmic trafficking of the OGF-OGFr complex and the NLS-dependent nuclear import pathway, the question can be raised as to the importance of this process to the functionality of this peptide-receptor axis. OGF is a tonically active inhibitory growth factor that is mediated by OGFr and targeted to the CKI pathway in order to modulate the cell cycle (Fig. 6B). To examine whether nuclear import of the OGF-OGFr complex is central to regulating cell proliferation, we utilized a combination of mutational analysis of the nuclear targeting signals with monitoring of DNA synthesis by incorporation of BrdU. These studies showed that cells transfected with OGFr-eGFP-containing mutations at all three NLS sites have more than three times the labeling index of cells transfected with wild-type OGFr-eGFP. Thus, abolishment of the NLS sites prevents the OGF-OGFr system from inhibiting DNA synthesis and, because this is a tonically active system, DNA synthesis is markedly increased.

The finding that transport of OGF-OGFr between the cytoplasmic and the nuclear compartments, and its dependence on NLSs, with respect to function, introduces the need to further define the pathways involved with these biological

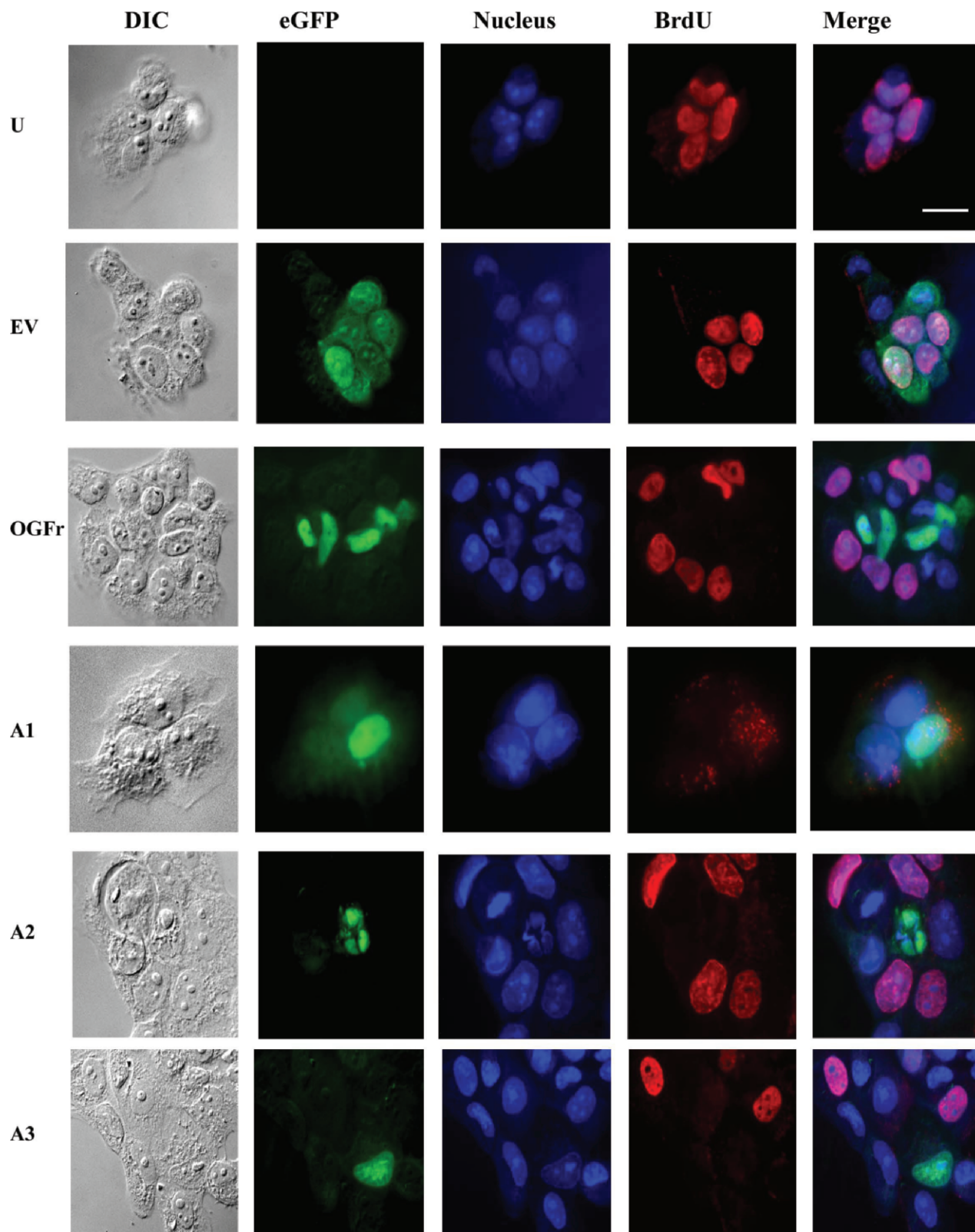


Figure 4. Trafficking of OGFr from the cytoplasm to the nucleus is reliant upon the integrity of the NLS. SCC-1 cells transfected with NLS-mutated OGFr-eGFP and treated with BrdU for 3 h prior to fixation 24 h after initiation of transfection. U, untransfected; EV, transfected with eGFP vector only; OGFr, wild-type OGFr-eGFP. Cells were visualized with differential interference optics (DIC), fluorescein (eGFP), UV (DAPI, Nucleus), and rhodamine (BrdU). Bar = 10 μ m. A color version of this figure is available in the online journal.

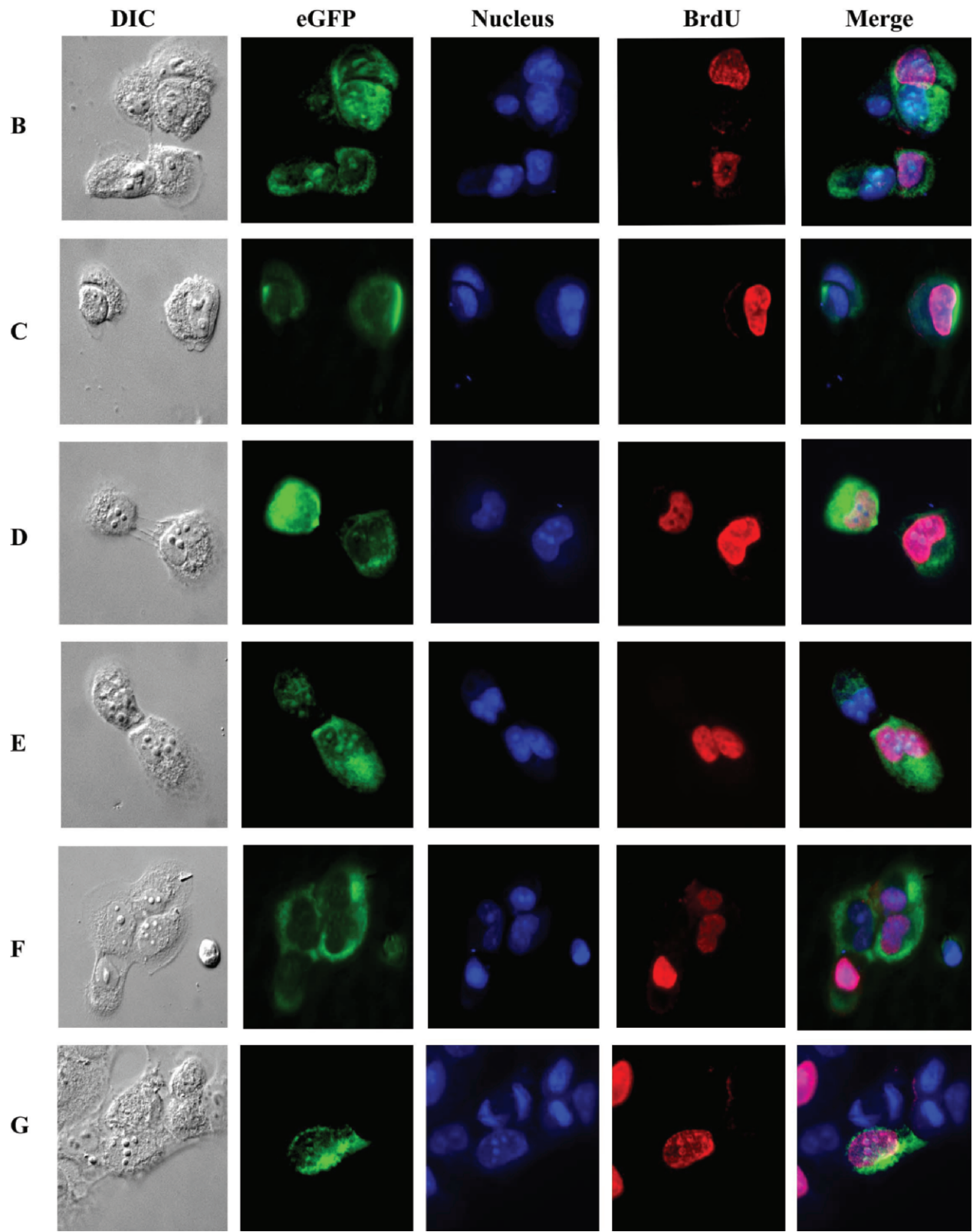


Figure 4. Continued.

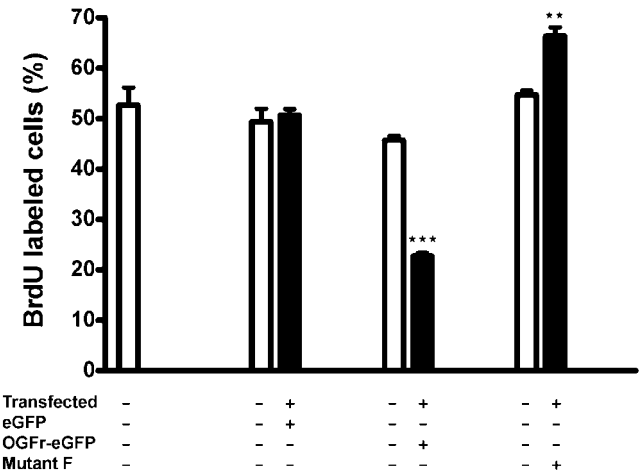


Figure 5. The inhibition of DNA synthesis by OGFr is reliant upon the integrity of the NLS. Bars (means $\pm$ SEM) represent the percentage of SCC-1 cells that were transfected with OGFr-eGFP or eGFP-vector only, or NLS Mutant F and expressing BrdU. For each condition, the number of BrdU-labeled cells was counted for transfected or untransfected cells. Statistically different from the untransfected wild-type group at $P < 0.01$ (**) and $P < 0.001$ (***).

processes. For example, nucleocytoplasmic trafficking is known to occur in a cascade of interactions that is dependent on a host of other factors and reactions (e.g., nucleoporins, karyopherins). In an earlier report (10, 11) using immuno-electron microscopy, karyopherin β , the largest group of transport receptors, has been found to be colocalized with OGFr in the paranuclear cytoplasm, perpendicular to the nuclear envelope in the perinuclear space, and in the nucleus. Thus, at least one component of the nuclear transport machinery is in place and interactive with OGFr. This begs the question of whether interfacing with karyopherin β is an essential element in the nucleocytoplasmic transport for the peptide-receptor complex. Moreover, it raises questions about the other components (e.g., karyopherin α , nucleoporins) that are determinants of the directionality of nuclear transport of OGFr. The results of the present study also bring forth questions about the repercussions of overexpression and reduced expression of those factors vital to nucleocytoplasmic trafficking of OGF and OGFr. For example, it may be predicted that reduced expression of karyopherin β would enhance cell proliferation because of the attenuation of OGF-OGFr inhibition of the cell cycle. In contrast, overexpression of karyopherin β

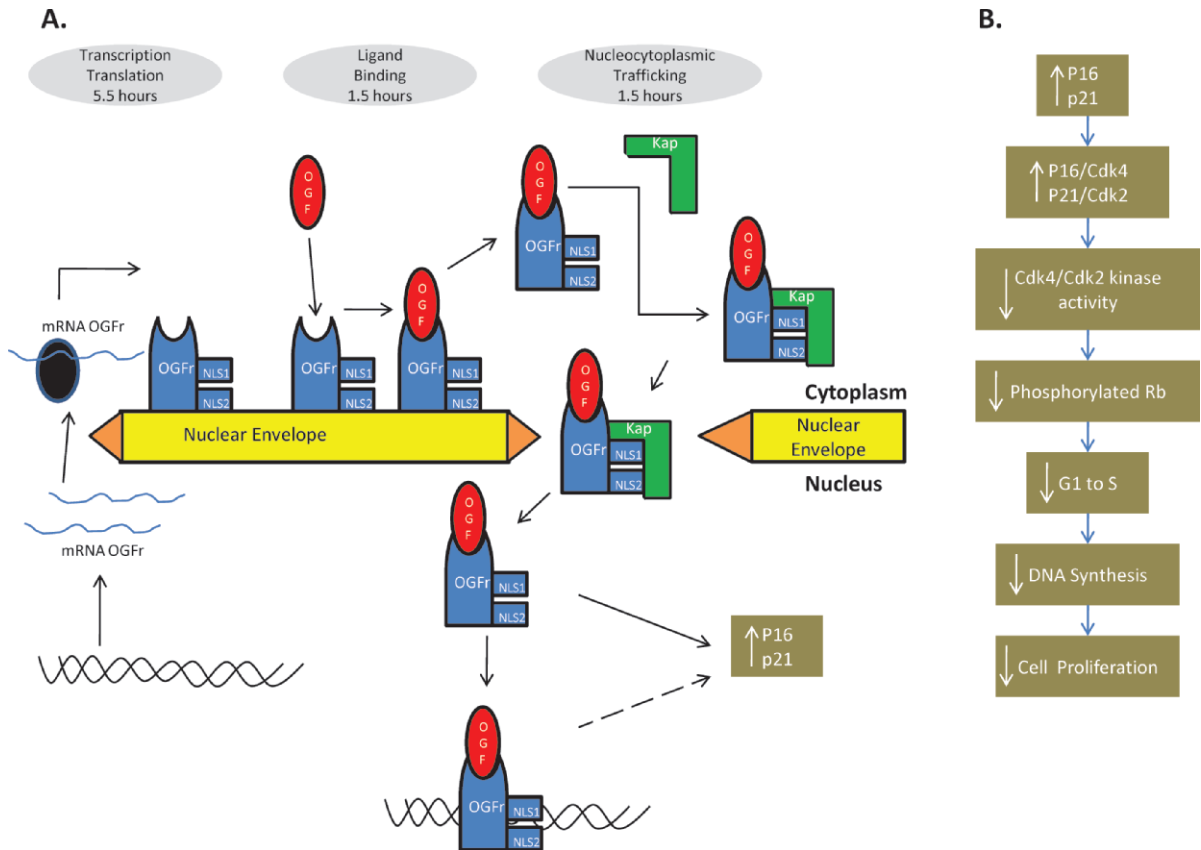


Figure 6. Schematic of a hypothetical model of the spatiotemporal (A) of OGF-OGFr nucleocytoplasmic trafficking, as well as subsequent events related to the signaling pathways of the cell cycle (B). The evidence for information in A emanates from information gathered herein, as well as from previous immunohistochemistry and immunoelectron microscopy studies (10, 11), whereas the signaling pathways involved with the OGF-OGFr axis and the cell cycle are documented in Cheng *et al.* (7–9). A color version of this figure is available in the online journal.

would be anticipated to slow cell proliferation because of the enhancement of OGF-OGFr regulation. Of course, overexpression and/or reduced expression of karyopherin may not alter cell proliferation, as there may be other regulatory devices that tie the amount of transport machinery to the number of OGF receptors translocated.

The clinical implications of our study speak to whether changes *in vivo* of the nucleocytoplasmic machinery related to the OGF-OGFr axis would be involved with understanding the etiology and pathogenesis of human disease or could be utilized for the treatment of human disorders. Deregulation of transport receptor shuttling pathways is known to occur in several disease states (15, 16, 19, 20). For example, overexpression of karyopherin β and/or karyopherin α family members is detected in colon, breast, and lung cancer (15). Moreover, deregulation of karyopherin $\alpha 2$ expression in melanoma cells and breast cancers correlates with poor survival and prognosis (15). Given the extraordinary biological control of the cell cycle by the OGF-OGFr axis, it may be envisioned that either a loss of or a gain in transport receptor shuttling pathways could contribute to an understanding about the onset and progression of a disease or the treatment of a disorder.

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