Original Research

Regulation of cell proliferation by the opioid growth factor receptor is dependent on karyopherin β and Ran for nucleocytoplasmic trafficking

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Abstract

The opioid growth factor (OGF; [Met^5]-enkephalin) and the OGF receptor (OGFr) form an endogenous and tonically active growth-regulating system that modulates cell proliferation by upregulating the cyclin-dependent kinase inhibitory pathway. Previous reports have documented that nucleocytoplasmic trafficking of the OGF–OGFr axis is dependent on nuclear localization signals. This study determined the specific transport factors required for the import of the OGF–OGFr complex from the cytoplasm to the nucleus using a probe of full-length OGFr fused to enhanced green fluorescent protein (eGFP) and knockdown of karyopherin α1, α2, α3, α4 or α6, karyopherin β1 or Ran with small interfering RNA (siRNA). A human squamous cell carcinoma of the head and neck cell line (squamous cell carcinoma-1, SCC-1) that was downregulated for karyopherin β1 or Ran did not have transport of OGFr-eGFP into the nucleus. Moreover, there was an increase of 44% in bromodeoxyuridine (BrdU)-labeled cells in cultures of SCC-1 that were transfected with siRNAs to karyopherin β1 or Ran compared with cells transfected with scrambled siRNA. No alteration in distribution of OGFr-eGFP or BrdU labeling indexes was recorded in cultures treated with siRNAs to karyopherin α1, α2, α3, α4 or α6. These results indicate that the regulation of cell proliferation by the OGF–OGFr axis is dependent on nucleocytoplasmic transport by karyopherin β1 as well as the gradient of RanGTP/RanGDP across the nuclear envelope, but is not reliant on adaptor molecules related to karyopherin α. Thus, the passage of the OGF–OGFr complex has controlled entry from the cytoplasm to the nucleus, and the timely and faithful translocation of this cargo across the nuclear envelope is critical to cell proliferation. These hierarchical levels of nuclear import provide multiple pathways for the subtle regulation of OGF–OGFr as it relates to the control of cell proliferative events.

Keywords: nucleocytoplasmic transport, karyopherin/importin, Ran, opioid growth factor receptor, opioid growth factor, siRNA, enkephalin, DNA synthesis, cell proliferation, green fluorescent protein

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Introduction

The movement of proteins from the cell cytoplasm into the nucleus is fundamental to cell biogenesis and division, physiological homeostasis, development, wound healing and disease.[1–3] Central to the transport process are members of the karyopherin β/importin β protein family – soluble transport factors that mediate import of cargoes into the nucleus and recognize signals (nuclear localization signals, NLS) displayed by cargo molecules.[1,4,5] Most karyopherin βs bind cargoes directly, although karyopherin α can serve as adaptors (the kapα isoforms) to recognize cargo. Directionality of transport for karyopherin β family members is accomplished by the small guanosine triphosphatase (GTPase) Ran.[6] Remarkably, the facilitated diffusion through the nuclear pore complex (NPC) that is mediated by karyopherins does not require metabolic energy or motor proteins but is driven by stochastic thermal motion.[7] The spatiotemporal control of transport receptor levels is not only important in nucleocytoplasmic trafficking in homoeostasis, but also has been linked to disease mechanisms.[1]

The endogenous opioid peptide, [Met^5]-enkephalin, was originally identified as a neuromodulator,[8,9] interacting with the δ-opioid receptor.[10] Subsequently, this peptide was discovered to be a tonically active regulator of cell proliferation[11] and, to distinguish its neural and non-neural distribution and biological function, was termed the opioid

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growth factor (OGF). OGF action is mediated by the non-classical opioid receptor – OGF receptor (OGFr). The gene for OGFr is located on chromosome 20q13.3 in humans.[12] The OGF–OGFr axis targets the p16/p21 cyclin-dependent kinase inhibitory pathway, thereby delaying the G_1–S phase of the cell cycle.[13–15] A series of observations have suggested that the OGF–OGFr complex translocates from the cytoplasm to the nucleus. First, both OGF and OGFr have been localized using immunohistochemistry in both the cytoplasm and the nucleus,[16–19] and receptor binding studies have determined the specific and saturable binding of OGFr in the nuclear rather than the cytoplasmic fractions.[11] Immunoelectron microscopic studies have revealed that OGFr is localized on the outer nuclear envelope, and both OGF and OGFr are co-localized in the cytoplasm and nucleus.[20,21] Moreover, OGFr was co-localized at the electron microscopic level of resolution with karyopherin β.[20] The gene for OGFr contains one bipartite and two monopartite NLS.[22] Using a probe of full-length human OGFr fused to enhanced green fluorescent protein (eGFP), along with mutational analysis, Cheng *et al.*[22] have shown that: (i) OGFr-eGFP undergoes a defined 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; (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.

The present investigation examined the transport system of the OGF–OGFr complex through the NPC. Using a tissue culture model of human squamous cell carcinoma of the head and neck (SCCHN), the relationship of karyopherin α and β transporters, along with the dependence on Ran, were studied by knockdown of these proteins and examination of the repercussions on OGFr-eGFP localization and DNA synthesis.

Materials and methods

Cell culture

SCC-1 cells were obtained from Dr Thomas E Carey, University of Michigan (Ann Arbor, MI, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Pennsylvania State University, Hershey, PA, USA), and supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 1.2% sodium bicarbonate and 5000 U/mL penicillin, 5 μg/mL streptomycin and 10 μg/mL neomycin. Cell cultures were maintained in a humidified atmosphere of 5% CO_2–95% air at 37°C. All experiments were conducted in triplicate.

Plasmid constructs

The OGFr constructs were generated by polymerase chain reaction (PCR) amplification of the human full-length isoform.[22] These cDNA fragments were then subcloned into the pEGFP N1 vector. The PCR primers used in each reaction incorporated *Eco*R1 and *Sal*1 restriction sites into the PCR products at the 5′ and 3′ ends, respectively. The primers used to perform the PCR amplifications were as follows: Zag152, 5′-GAATTCTAGCCGAGCATGGACGACC-3′ and Zag153, 5′-GTCGACTTCCAGACTTGGCAGAAGACT-3 (restriction sites are underlined). The PCR products were ethanol precipitated, digested and ligated into the pEGFP N1 vector.

OGFr-eGFP transfections

Five micrograms of plasmid DNA and 5 μL of Lipofectamine2000 were diluted in 250 μL of serum-free, antibiotic-free DMEM, and 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 followed by incubation for four hours at 37°C, after which the reagent was aspirated and the cells incubated in serum-supplemented, antibiotic-free DMEM.

Small interfering RNA transfection

Karyopherin β1, α1, α2, α3, α4 or α6, or Ran, targeted small interfering RNA (siRNAs) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and negative control siRNAs were purchased from Ambion (Austin, TX, USA). In all, 2×10^5 cells/well were seeded into 35 mm glass-bottom culture dishes (MatTek, Ashland, MA, USA) containing 1 mL of media without antibiotics for 24 h. Cells were transfected with 20 nmol/L siRNA using oligofectamine reagent (Invitrogen, Carlsbad, CA, USA) in serum- and antibiotic-free media for 24 h, followed by the addition of 1 mL of fresh media without antibiotics. Seventy-two hours after seeding, cells were transfected with OGFr-eGFP.

For transfection in 24-well plates containing 22 mm coverslips, 8×10^4 cells/well were seeded in 500 μL of media without antibiotics. Cells were transfected with 20 nmol/L siRNA with oligofectamine reagent in serum- and antibiotic-free media. At 24 h, 500 μL of fresh media without antibiotics was added to the cultures and, at 48 h after initial transfection, cells were transfected with OGFr-eGFP for four hours.

Immunostaining, bromodeoxyuridine and microscopy

The subcellular localization of OGFr-eGFP in living SCC-1 cells was analyzed by fluorescent microscopy with deconvolution optics (Olympus IX81, Center Valley, PA, USA) after 24 h of transfection. In brief, the cells were stained with Hoechst's for five minutes in order to visualize nuclei, and washed twice with media prior to observation. At least 150 cells/group were counted in a masked manner.

Karyopherin β1 and subcellular localization of OGFr-eGFP were analyzed by immunostaining. SCC-1 cells transfected with karyopherin β1 siRNA growing on 22 mm^2 coverslips were transfected for four hours with OGFr-eGFP and incubated in serum-supplemented DMEM for an additional 24 h. Cells were washed twice with 1× phosphate-buffered saline (PBS) before and after fixing with ice-cold methanol:acetone (1:1) at −20°C for 10 min. Cells were blocked with 1% bovine serum

albumin (BSA) (EMD Chemical, Gibbstown, NJ, USA) in PBS for 30 min. After blocking, mouse anti-karyopherin β (BD Transduction Laboratories, San Diego, CA, USA) and rabbit Alexa-488-conjugated anti-GFP antibody (Invitrogen, Carlsbad, CA, USA) were added in 1% BSA–PBS and incubated for 60 min at 20°C. Following a wash with PBS, cells were incubated with Alexa-568-conjugated anti-mouse immunoglobulin G for 60 min at 20°C. Cells were stained with 4′-6-diamidino-2-phenylindole (DAPI) (1 μg/mL) and sealed onto glass slides. At least 150 cells/group that had cytoplasmic staining only relative to the total number of nuclei (DAPI stained) were counted in a masked manner.

Bromodeoxyuridine (BrdU) incorporation and subcellular localization of OGFr-eGFP were analyzed by immunostaining. SCC-1 cells transfected with different siRNAs growing on 22 mm^2 coverslips were transfected for four hours with OGFr-eGFP and incubated in serum-supplemented DMEM for an additional 20 h. Fresh complete DMEM with 30 μmol/L BrdU (Sigma-Aldrich, St Louis, MO, USA) was added to each culture for three hours, and cells stained according to Donahue *et al.*[16] Cells were stained with DAPI (1 μg/mL) and sealed onto glass slides. At least 150 cells/group were counted in a blinded manner.

Western blot analysis

siRNA-transfected cells from each treatment were solubilized in 100 μL radio immunoprecipitation assay buffer (1× PBS, 10 μmol/L IGEPAL [polyoxyethylene nonyl phenol], 1 mg/mL sodium dodecyl sulfate [SDS]), containing complete miniprotease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). Total proteins (40 μg) were subjected to 12% SDS-polyacrylamide gel electrophoresis (PAGE), followed by transfer to polyvinylidene difluoride (Millipore, Billerica, MA, USA) using standard protocols[13–15] and probed with antibodies (1:200) to karyopherin β1, α1, α2 and α4, and Ran (Santa Cruz Biotechnology) or karyopherin α3 (Abcam Inc, Cambridge, MA, USA), as well as an antibody (1:50) to karyopherin α1/6 (Sigma-Aldrich). Blots were washed and incubated with secondary anti-mouse horseradish peroxidase-conjugated antibodies (GE Healthcare-Amersham Biosciences, Piscataway, NJ, USA) or anti-goat horseradish peroxidase-conjugated antibodies (Santa Cruz Biotechnology), and developed using a chemiluminescence Western blotting detection system (GE Healthcare-Amersham Biosciences).

In order to determine equal loading of total protein samples, blots were reprobed with monoclonal antibody against β-actin (Sigma-Aldrich) at a dilution of 1:2000. If necessary, membranes were stripped in stripping buffer (62.5 mmol/L Tris-HCl and 100 mmol/L β-mercaptoethanol/2% SDS, pH 6.7) at 50°C before being reprobed.

To quantify expression levels, the optical density of each band was determined by densitometry, and analyzed by QuickOne (Bio-Rad Laboratories, Hercules, CA, USA). Each value was normalized to β-actin from the same blot. At least three blots from independent experiments were utilized.

Statistical analysis

Values were assessed by one-way analysis of variance and Newman–Keul's postmultiple comparison tests. Differences were considered statistically significant when $P < 0.05$.

Results

Downregulation of karyopherin β1 blocks nuclear import of OGFr

To establish the role of karyopherin β1 in OGFr nuclear import, the effects of siRNA-targeted karyopherin β1 were examined by Western blotting at 72 h after transfection with siRNA (Figure 1a). The expression of karyopherin β1 in karyopherin β1 siRNA-transfected cells was downregulated 59% compared with that of scrambled siRNA-transfected cells.

Karyopherin β1 depletion markedly affected the location of OGFr-eGFP in SCC-1 cells in contrast to the distribution in control preparations (i.e. scrambled siRNA-transfected cells) (Figure 1b). Whereas 4% of the cells subjected to scrambled siRNA had OGFr-eGFP only in the cytoplasm, 50% of the cells in the karyopherin β1 siRNA cultures had OGFr-eGFP only in the cytoplasm (Figure 1c).

Downregulation of Ran blocks nuclear import of OGFr

To test the role of Ran in OGFr nuclear import, the effects of siRNA-targeted Ran were examined by Western blotting at 72 h after transfection with siRNA (Figure 2a). The expression of Ran in Ran siRNA-transfected cells was downregulated approximately 70% compared with that of scrambled siRNA-transfected cells.

Depletion of Ran markedly altered the morphological distribution of OGFr-eGFP in SCC-1 cells in contrast to the location of OGFr-eGFP in control preparations (i.e. scrambled siRNA-transfected cells) (Figure 2b). Whereas 1% of the cells subjected to scrambled siRNA had OGFr-eGFP only in the cytoplasm, 55% of the cells in the Ran siRNA cultures had OGFr-eGFP only in the cytoplasm (Figure 2c).

Downregulation of karyopherin α proteins do not block nuclear import of OGFr

To evaluate the relationship of karyopherin α proteins in the import of OGFr, siRNA-targeted karyopherin α proteins were utilized in Western blotting of transfected cells at 72 h (Figure 3a). Transfection with siRNA to karyopherin α1, α2, α3, α4 or α6 downregulated each protein by 47–91% in comparison to that of scrambled siRNA-transfected cells.

Depletion of each of the karyopherin α proteins with respect to the location of OGFr-eGFP in the cells had no effect on OGFr-eGFP distribution (Figure 3b). Cells subjected to karyopherin α siRNA had 1–4% of OGFr-eGFP located only in the cytoplasm, and this was similar to that of 1% of the cells treated with scrambled siRNA (Figure 3c).

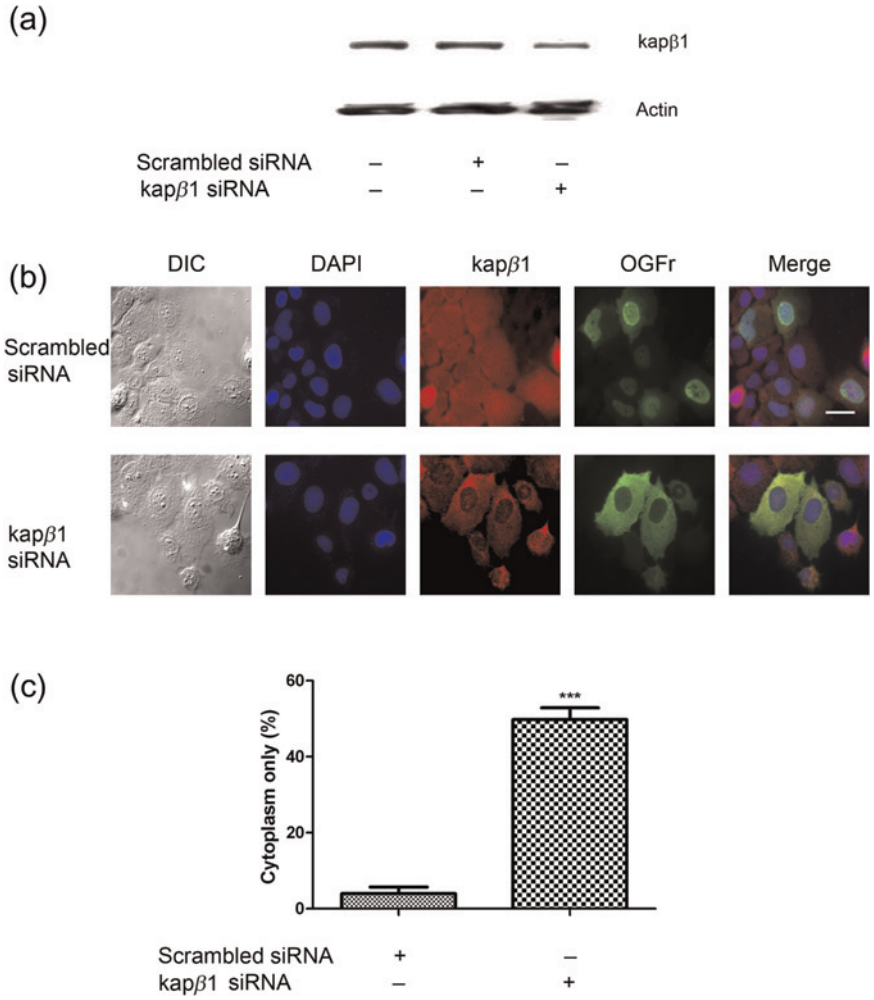

Figure 1 Downregulation of karyopherin β1 blocks nuclear import of OGFr. (a) Western blotting analysis demonstrating the specificity of kapβ1 knockdown in SCC-1 cells. Log-phase cells were transfected for 24 h with either scrambled siRNA or kapβ1 siRNA. Seventy-two hours after transfection, cells were harvested and proteins isolated. (b) SCC-1 cells were transfected for 24 h with either scrambled siRNA or kapβ1 siRNA and, 48 h later, transfected with OGFr-eGFP for four hours. At 96 h in culture, cells were immunostained with antibodies to kapβ1. Cells were visualized with differential interference optics (DIC), DAPI (nucleus), rhodamine (kapβ1) and fluorescein (OGFr-eGFP). Bar = 10 μm. (c) The subcellular distribution of OGFr-eGFP after kapβ1 or scrambled siRNA transfection. Data represent mean (%) $\pm$ standard error of cells with only staining of the cytoplasm. ***Significantly different from scrambled siRNA-transfected cells at $P < 0.001$. OGF, opioid growth factor; OGFr, OGF receptor; kapβ1, karyopherin β1; SCC-1, squamous cell carcinoma-1; siRNA, small interfering RNA; eGFP, enhanced green fluorescent protein; DAPI, 4′-6-diamidino-2-phenylindole (A color version of this figure is available in the online journal)

Regulation of cell proliferation by OGFr is dependent on karyopherin β1 and Ran

To determine whether karyopherin β1, Ran or karyopherin α1, α2, α3, α4 or α6 are important to the inhibitory function of the OGF–OGFr axis, we examined DNA synthesis after transfection with siRNAs to each of these proteins (Figure 4). In contrast to a labeling index of 50% in cells transfected with scrambled siRNA, cells treated with karyopherin β1 or Ran siRNAs had a labeling index of 72% (a 44% increase). However, cells subjected to karyopherin α1, α2, α3, α4 or α6 siRNAs had labeling indexes ranging from 46% to 56%, and there were no differences from that of cells exposed to scrambled siRNA.

Discussion

This study shows for the first time that the OGF–OGFr axis, an inhibitory biological system, is dependent on nucleocytoplasmic transport factors karyopherin β1 and Ran, but not karyopherin α1, α2, α3, α4 or α6, for its function as a suppressor of cell proliferation. Several lines of evidence support this conclusion. First, attenuation of karyopherin β1 using siRNA technology blocked the transport of OGFr-eGFP into the nucleus, and sequestered OGFr-eGFP in the cytoplasm. These results indicate that without intact karyopherin β1, the OGF–OGFr system cannot translocate from the cytoplasm into the nucleus. Second, knockdown of Ran interfered with the translocation of OGFr-eGFP from the cytoplasm to the nucleus. These data indicate that disruption of the gradient of RanGTP/RanGDP across the nuclear envelope constitutes a driving force for protein trafficking through the NPC,[1,2,4–6] and consequently obstruction of this directionality is important to the passage of the OGF–OGFr complex from the cytoplasm to the nucleus. Third, knockdown of the adaptor molecules α1 α2, α3, α4 or α6 does not influence nucleocytoplasmic transport efficiency, thereby showing that at

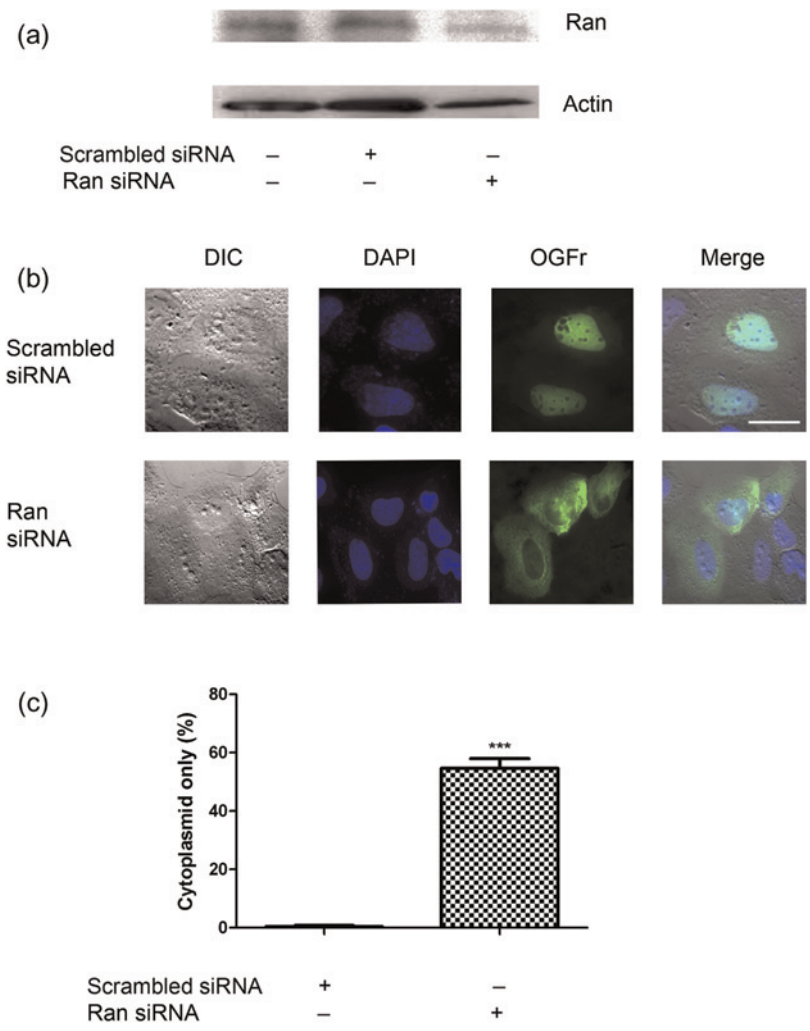

Figure 2 Downregulation of Ran blocks nuclear import of OGFr. (a) Western blotting analysis demonstrating the specificity of Ran knockdown in SCC-1 cells. Log-phase cells were transfected for 24 h with either scrambled siRNA or Ran siRNA. Seventy-two hours after transfection, cells were harvested and proteins isolated. (b) SCC-1 cells were transfected for 24 h with either scrambled siRNA or Ran siRNA and, 48 h later, transfected with OGFr-eGFP for four hours. Cells were visualized with differential interference optics (DIC), DAPI (nucleus) and fluorescein (OGFr-eGFP). Bar = 10 μm. (c) The subcellular distribution of OGFr-eGFP after Ran or scrambled siRNA transfection. Data represent mean (%) $\pm$ standard error of cells with only staining of the cytoplasm. ***Significantly different from scrambled siRNA-transfected cells at $P < 0.001$. OGF, opioid growth factor; OGFr, OGF receptor; SCC-1, squamous cell carcinoma-1; siRNA, small interfering RNA; eGFP, enhanced green fluorescent protein; DAPI, 4′-6-diamidino-2-phenylindole (A color version of this figure is available in the online journal)

least any of these five homologs of karyopherin α are not responsible for the shuttling of the OGF–OGFr complex to docking at the NPC and translocation to the nucleoplasm. Fourth, interruption of the NLS and karyopherin β1, or the recycling of RanGDP/RanGTP, increases the number of cells proliferating, indicating the essential significance of these molecules to not only OGF–OGFr passage from the nucleus to the cytoplasm, but also to the eventual effect on the cell cycle. Finally, consonant with our finding that members of the karyopherin α family are not involved with OGF–OGFr transport, DNA synthesis was not influenced by knockdown of karyopherin α1, α2, α3, α4 or α6. Thus, the precise sequence and the structural context of the NLS of OGFr define a specificity for karyopherin β1 and, in concert with a dependence on Ran, govern the regulatory pathway of the OGF–OGFr axis in cell proliferation.

The present study examined karyopherin β1, the karyopherin β family member that has received the most attention,[1–5] with respect to being a transport factor for the OGF–OGFr axis. However, it should be recognized that the karyopherin β family is comprised of more than 20 members in mammals.[4,23] Likewise, although we have studied five members of the karyopherin α family, there are at least six identified karyopherin α isoforms in humans.[23,24] It is known that several karyopherins, despite their low sequence similarity, can recognize the same cargo. For example, at least four karyopherin pathways are used to import core histones in both yeast and mammalian cells.[4,24] Thus, the complexities of the interactions of karyopherin with their cargoes, and in this case OGFr, raises questions about other karyopherins – β and α – that play a role in cytoplasmic to nuclear shuttling of this peptide–receptor complex. Therefore, further work on identifying other karyopherins in OGF–OGFr nucleocytoplasmic transport are needed, as are structural studies that enable us to understand how each karyopherin interacts with the OGF–OGFr cargo.

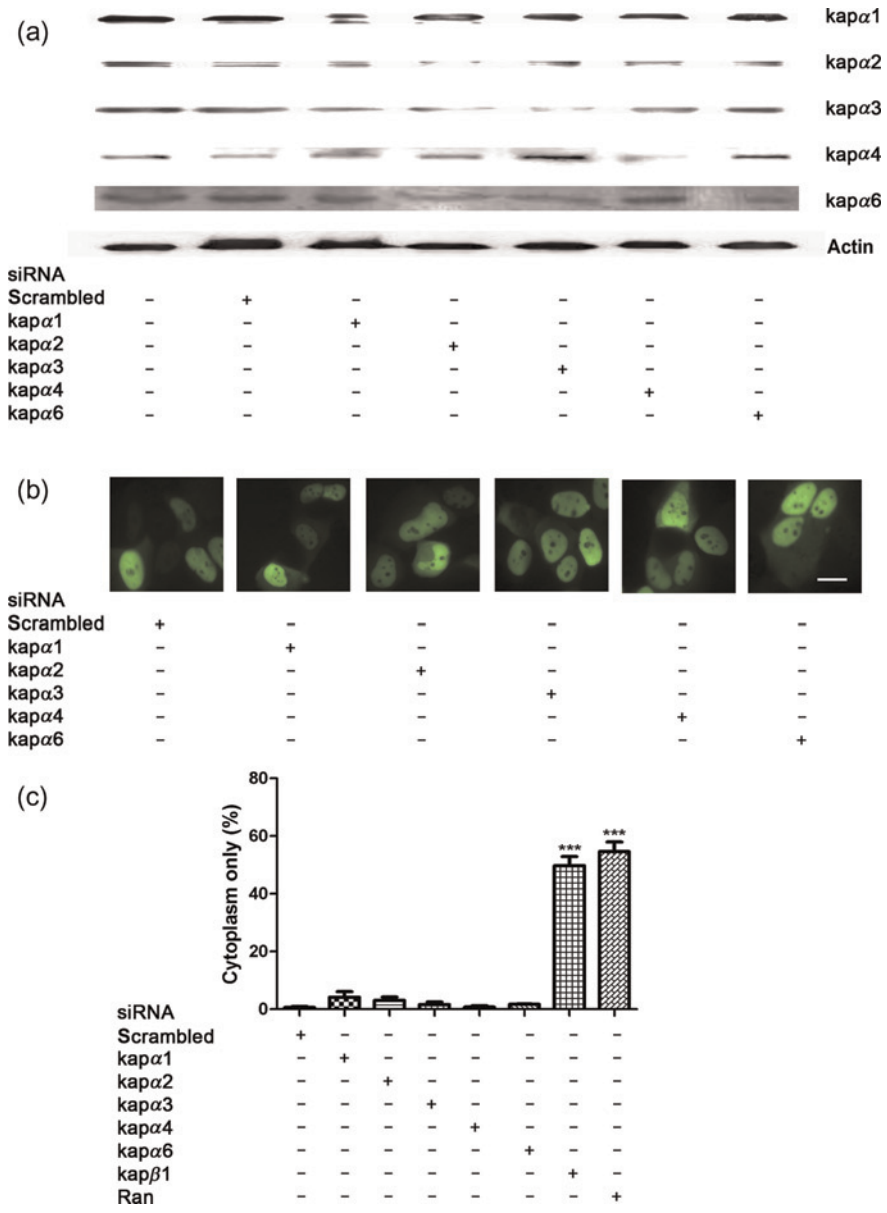

Figure 3 Downregulation of kapα1, α2, α3, α4 or α6 does not block nuclear import of OGFr. (a) Western blotting analysis demonstrating the specificity of knockdown of kapα1, α2, α3, α4 or α6 in SCC-1 cells. Log-phase cells were transfected for 24 h with either scrambled siRNA or kapα1, α2, α3, α4 or α6 siRNAs. Seventy-two hours after transfection, cells were harvested and proteins isolated. (b) SCC-1 cells were transfected for 24 h with either scrambled siRNA or kapα1, α2, α3, α4 or α6 siRNAs and, 48 h later, transfected with OGFr-eGFP for four hours, and preparations visualized. Bar = 10 μm. (c) The subcellular distribution of OGFr-eGFP after kapα1, α2, α3, α4, α6 or scrambled siRNA; data from Figures 1c and 2c for kapβ1 and Ran, respectively, are included for comparison. Data represent mean (%) $\pm$ standard error of cells with only staining of the cytoplasm. ***Significantly different from scrambled siRNA-transfected cells at $P < 0.001$. OGF, opioid growth factor; OGFr, OGF receptor; kap, karyopherin; SCC-1, squamous cell carcinoma-1; siRNA, small interfering RNA; eGFP, enhanced green fluorescent protein (A color version of this figure is available in the online journal)

Taken together with previous reports regarding gene/protein structure of NLS sequences, epi-fluorescent and immunoelectron microscopic findings, and cell/molecular biological data regarding OGF and OGFr,[13–22] we now have a clearer perspective of the nucleocytoplasmic relationships of the OGF–OGFr axis in regulating cell proliferation (Figure 5). OGFr is synthesized and located on the outer nuclear envelope, and OGF that gains entrance to the cell by active transport (Cheng and colleagues, unpublished observations) binds to OGFr wherein this peptide–receptor complex is released at the outer nuclear envelope and is located in the paranuclear cytoplasm. In this report, we have learned that karyopherin β1 which binds to two of the three NLSs in SCC-1 encoded by OGFr,[22] as well as RanGDP, guide the OGF–OGFr complex to – and through – the NPC into the nucleus. Although those molecules in the NPC that are essential for passage of OGF–OGFr require clarification, as well as the relationship between this complex and chromatin, previous reports have documented that p16 and/or p21 is(are) elevated and lead to a delay in the G1–S phase of the cell cycle. Thus, the present findings and previous reports reveal that there are multiple avenues for the regulation of the OGF–OGFr system with respect to cell proliferation. Diseases and

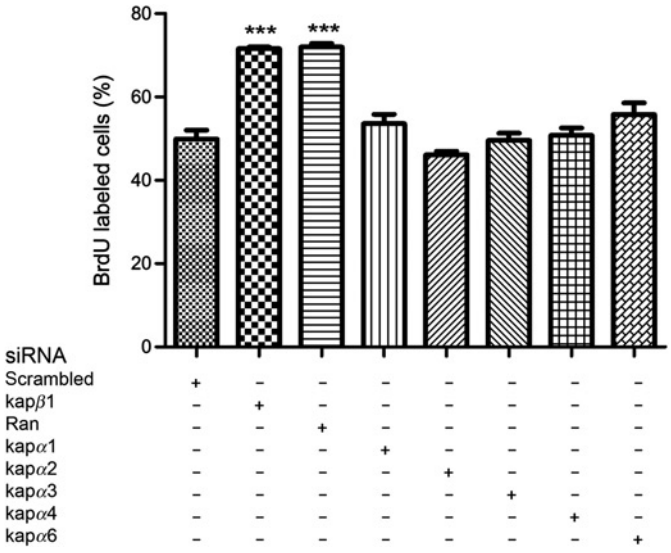

Figure 4 Inhibition of DNA synthesis by OGFr is reliant upon the integrity of kapβ1 and Ran. Bars (means $\pm$ standard error) represent the percentage of bromodeoxyuridine-labeled cells in cultures that were transected with kapβ1, α1, α2, α3, α4, α6, Ran or scrambled siRNA. ***Statistically different from the scrambled siRNA-transfected group at $P < 0.001$. OGF, opioid growth factor; OGFr, OGF receptor; kap, karyopherin

irregularities that impact any of these factors may change the pace of cell proliferation. For example, defects in karyopherin β1 would attenuate the OGF–OGFr axis and cell proliferation would be accelerated. Likewise, exaggeration of some of these pathways would result in a decrease in cell proliferation.

Several studies have documented that transport might be regulated by changes in receptor or adaptor expression levels.[1] Most relevant to the OGF–OGFr system, deregulation of transport shuttling pathways occurs in several disease states.[1,2–4,25] The overexpression of karyopherin β and/or α has been detected in breast, colon and lung cancers.[26,27] In fact, increased nuclear import that correlates with poor survival and prognosis has been recorded with karyopherin α2 overexpression in both melanoma cells and breast cancers.[27,28] Mislocalization of proteins due to alterations in nucleocytoplasmic trafficking have also been observed.[4] Nuclear mislocalization of the transcriptional activator nuclear factor-κB has been reported in breast, ovary, colon and thyroid tumor cells,[29,30] and in vascular smooth muscle cells, endothelial cells and macrophages of atherosclerotic plaques.[29] Changes to the import receptors themselves can also cause detrimental effects to the cell. A truncated form of karyopherin α1 isolated in a breast

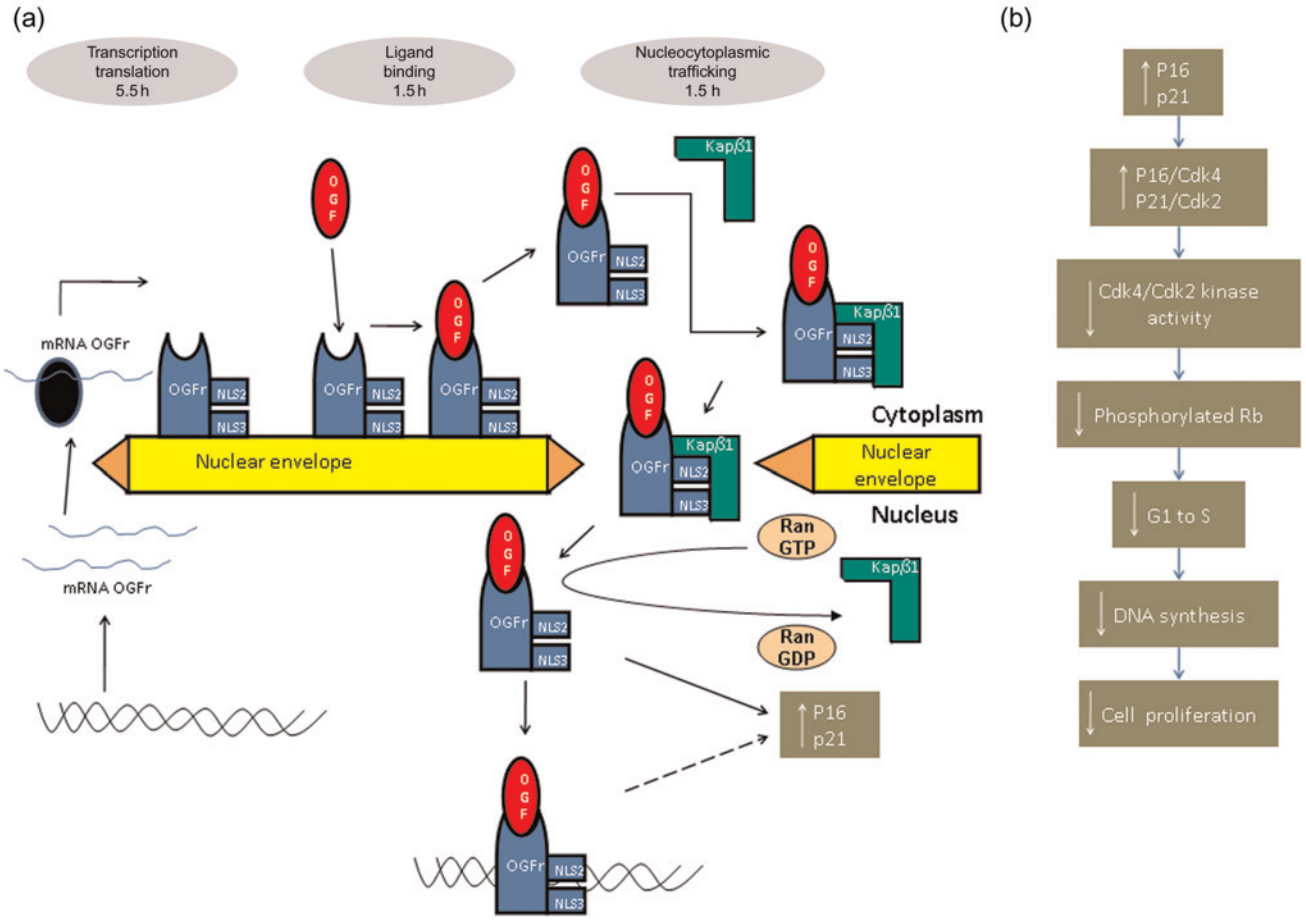

Figure 5 Schematic of a hypothetical model of the spatiotemporal pattern of OGF–OGFr nucelocytoplasmic trafficking (a) as well as subsequent events related to the signaling pathways of the cell cycle (b). The evidence for this model emanates from data gathered herein as well as from previous immunohistochemistry and immunoelectron microscopy studies.[20,21] This drawing is modified from that of previous reports.[20,22,40] NLS, nuclear localization signal; OGF, opioid growth factor; OGFr, OGF receptor; kap, karyopherin (A color version of this figure is available in the online journal)

cancer cell line has been linked to a defect in p53 nuclear import,[31] resulting in constitutive cytoplasmic localization of p53 with repression of genes involved in apoptosis and increased expression of those involved in cell proliferation.[32,33] Therefore, transport of macromolecules between the nucleus and cytoplasm is a critical cellular process for eukaryotes, and the machinery is subject to multiple levels of control. Localization of these proteins in the wrong subcellular compartments could result in pathological states.[1,2]

The utilization of exogenous OGF in the treatment of patients with advanced pancreatic cancer has been reported,[34,35] and the safety and efficacy of this therapy established. Based on the importance of the OGF–OGFr system in homeostasis of cellular renewal and the modulation of disease processes as shown in animals and humans,[1,36–39] understanding the pathway(s) of OGF–OGFr action is vital in not only elucidating the etiology and pathogenesis of irregularities in this system, but also in utilizing the OGF–OGFr axis for therapy. The present data reveal that controlled nuclear entry by the OGF–OGFr complex, and the timely translocation of the peptide–receptor cargo, are especially critical for correct function. Furthermore, we have observed that deregulation or 'hijacking' of transport pathways is also central to the efficacy of OGF–OGFr action. Therefore, patients with abnormalities of karyopherin β1 or Ran may not be candidates for treatment with OGF. Moreover, whether defects in karyopherin β1 or Ran are a primary or secondary factor in disease pathogenesis by disabling a critical inhibitory pathway (i.e. OGF–OGFr) regulating cell proliferation needs to be clarified.

Author contributions: ISZ conceptually designed, conducted and interpreted the experimental work and wrote the manuscript; FC performed the experiments and contributed to data analysis; and PJM designed experiments, interpreted the experimental work and wrote the manuscript.

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REFERENCES

1 Terry LJ, Shows B, Wente SR. Crossing the nuclear envelope: hierarchical regulation of nucelocytoplasmic transport. *Science* 2007;**318**:1412–16
2 Chahine MN, Pierce GN. Therapeutic targeting of nuclear protein mport in pathological cell conditions. *Pharmacol Rev* 2009;**61**:358–72
3 McLane LM, Corbett AH. Nuclear localization signals and human disease. *IUBMB Life* 2009;**61**:697–706
4 Mosammaparast N, Pemberton LF. Karopherins: from nuclear-transport mediators to nuclear-function regulators. *Trends Cell Biol* 2004;**14**:547–56
5 Wagstaff KM, Jans DA. Importins and beyond: non-conventional nuclear transport mechanisms. *Traffic* 2009;**10**:1188–98
6 Yudin D, Fainsilber M. Ran on tracks – cytoplasmic roles for a nuclear regulator. *J Cell Sci* 2009;**122**:587–93
7 Peters R. Functionalization of a nanopore: the nuclear pore complex paradigm. *Biochem Biophys Acta* 2009;**1793**:1533–9
8 Hughes J, Smith TW, Kosterlitz HW, Fothergill LA, Morgan BA, Morris HR. Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature* 1975;**258**:577–80
9 Akil H, Watson SJ, Young E, Lewis ME, Khachaturian H, Walker JM. Endogenous opioids: biology and function. *Ann Rev Neurosci* 1984;**7**:223–55
10 Leslie FM. Methods used for the study of opioid receptors. *Pharmacol Rev* 1987;**39**:197–249
11 Zagon IS, Verderame MF, McLaughlin PJ. The biology of the opioid growth factor receptor (OGFr). *Brain Res Rev* 2002;**38**:351–76
12 Zagon IS, Verderame MF, Allen SS, McLaughlin PJ. Cloning, sequencing, chromosomal location, and function of a cDNA encoding the opioid growth factor receptor (OGFr) in humans. *Brain Res* 2000;**856**: 75–83
13 Cheng F, Zagon IS, Verderame MF, McLaughlin PJ. The OGF–OGFr axis utilizes the p16 pathway to inhibit progression of human squamous cell carcinoma of the head and neck. *Cancer Res* 2007;**67**:10511–18
14 Cheng F, McLaughlin PJ, Verderame MF, Zagon IS. The OGF–OGFr axis utilizes the p21 pathway to restrict progression of human pancreatic cancer. *Mol Cancer* 2008;**7**:5–17
15 Cheng F, McLaughlin PJ, Verderame MF, Zagon IS. The OGF–OGFr axis utilizes the $p16^{INK4a}$ and $p21^{WAF1/CIP1}$ pathways to restrict normal cell proliferation. *Mol Biol Cell* 2009;**20**:319–27
16 Donahue RN, McLaughlin PJ, Zagon IS. Cell proliferation of human ovarian cancer is regulated by the opioid growth factor–opioid growth factor receptor axis. *Am J Physiol* 2009;**296**:R1716–25
17 Goldenberg D, Zagon IS, Fedok F, Crist H, McLaughlin PJ. The presence of the opioid growth factor (OGF)–OGF receptor (OGFr) axis in human non-medullary thyroid cancer. *Thyroid* 2008;**18**:1165–70
18 Levin RJ, Wu Y, McLaughlin PJ, Zagon IS. Expression of the opioid growth factor, [Met^5]-enkephalin, and the zeta opioid receptor in head and neck squamous cell carcinoma. *Laryngoscope* 1997;**107**:335–9
19 McLaughlin PJ, Levin RJ, Zagon IS. Regulation of human head and neck squamous cell carcinoma growth in tissue culture by opioid growth factor. *Int J Oncol* 1999;**14**:991–8
20 Zagon IS, Ruth TB, McLaughlin PJ. Nucleocytoplasmic distribution of opioid growth factor and its receptor in tongue epithelium. *Anat Rec A* 2005;**282**:24–37
21 Zagon IS, Ruth TB, Leure-duPree AE, Sassani JW, McLaughlin PJ. Immunoelectron microscopic localization of the opioid growth factor receptor (OGFr) and OGF in the cornea. *Brain Res* 2003;**967**: 37–47
22 Cheng F, McLaughlin PJ, Verderame MF, Zagon IS. Dependence on nuclear localization signals of the opioid growth factor receptor in the regulation of cell proliferation. *Exp Biol Med* 2009;**234**:532–41
23 Frankel MB, Knoll LJ. The ins and outs of nuclear trafficking: unusual aspects in Apicomplexan parasites. *DNA Cell Biol* 2009;**28**:277–84
24 Quensel C, Friedrich B, Sommer T, Hartmann E, Kohler M. *In vivo* analysis of importin alpha proteins reveals cellular proliferation inhibition and substrate specificity. *Mol Cell Biol*, 2004;**24**:10246–55
25 Borlido J, Zecchini V, Mills IG. Nuclear trafficking and functions of endocytic proteins implicated in oncogenesis. *Traffic* 2009;**10**: 1209–20
26 Kau TR, Way JC, Silver PA. Nuclear transport and cancer: from mechanism to intervention. *Nat Rev Cancer* 2004;**4**:106–17
27 Dahl E, Kristiansen G, Gottlob K, Klaman I, Ebner E, Hinzman B, Hermann K, Pilarsky C, Durst M, Klinkhammer-Schalke M, Blaszyk H, Knuechel R, Hartmann A, Rosenthal A, Wild PJ. Molecular profiling of laser-microdissected matched tumor and normal breast tissue identifies karyopherin α2 as a potential novel prognostic marker in breast cancer. *Clin Cancer Res* 2006;**12**:3950–60
28 Winnepenninckx V, Lazar V, Michiels S, Dessen P, Stas M, Alonso SR, Avril M-F, Romero PLO, Robert T, Balacescu O, Eggermont AMM, Lenoir G, Sarasin A, Tursz T, van den Oord JJ, Spatz A. Gene expression profiling of primary cutaneous melanoma and clinical outcome. *J Natl Cancer Inst* 2006;**98**:472–82
29 Brand K, Page S, Rogler G, Bartsch A, Brandl R, Knueschel R, Page M, Kaltschmidt C, Baeuerle PA, Neumeier D. Activated transcription factor nuclear-kappa B is present in the atherosclerotic lesion. *J Clin Invest* 1996;**97**:1715–22
30 Lee CH, Jeon YT, Kim SH, Song YS. NF-kappaB as a potential molecular target for cancer therapy. *Biofactors* 2007;**29**:19–35

31 Kim I-S, Kim D-H, Han S-M, Chin M-U, Nam H-J, Cho H-P, Choi S-Y, Song B-J, Kim E-R, Bae Y-S, Moon Y-H. Truncated form of importin α identified in breast cancer cell inhibits nuclear import of p53. *J Biol Chem* 2000;**275**:23139–45
32 Moll UM, Riou G, Levine A. Two distinct mechanisms alters p53 in breast cancer: mutation and nuclear exclusion. *Proc Natl Acad Sci USA* 1992;**89**:7262–6
33 Moll UM, LaQuaglia M, Benard J, Riou G. Wild-type p53 protein undergoes cytoplasmic sequestration in undifferentiated neuroblastomas but not in differentiated tumors. *Proc Natl Acad Sci USA* 1995;**92**:4407–11
34 Smith JP, Conter RL, Bingaman SI, Harvey HA, Mauger DT, Ahmad M, Demers LM, Stanley WB, McLaughlin PJ, Zagon IS. Treatment of advanced pancreatic cancer with opioid growth factor: phase I. *Anti-Cancer Drugs* 2004;**15**:203–9
35 Smith JP, Bingaman SI, Mauger DT, Harvey HA, Demers LM, Zagon IS. Opioid growth factor (OGF) improves clinical benefit and survival in patients with advanced pancreatic cancer. *Open Access J Clin Trials* 2010;**2**:1–12
36 Jaglowski JR, Zagon IS, Stack BC, Verderame MF, Leure-duPree AE, Manning JD, McLaughlin PJ. Opioid growth factor (OGF) enhances tumor growth inhibition and increases the survival of paclitaxel-treated mice with squamous cell carcinoma of the head and neck. *Cancer Chemother Pharmacol* 2005;**56**:97–104
37 McLaughlin PJ, Jaglowski JR, Verderame MF, Stack BC, Leure-duPree AE, Zagon IS. Enhanced growth inhibition of squamous cell carcinoma of the head and neck by combination therapy of paclitaxel and opioid growth factor. *Int J Oncol* 2005;**26**:809–16
38 Zagon IS, Jaglowski JR, Verderame MF, Smith JP, Leure-duPree AE, McLaughlin PJ. Combination chemotherapy with gemcitabine and biotherapy with opioid growth factor (OGF) enhances the growth inhibition of pancreatic adenocarcinoma. *Cancer Chemother Pharmacol* 2005;**56**:510–20
39 McLaughlin PJ, Levin RJ, Zagon IS. Opioid growth factor (OGF) inhibits the progression of human squamous cell carcinoma of the head and neck transplanted into nude mice. *Cancer Lett* 2003;**199**:209–17
40 Cheng F, McLaughlin PJ, Banks WA, Zagon IS. Passive diffusion of naltrexone into human and animal cells and upregulation of cell proliferation. *Am J Physiol Regul Integr Comp Physiol* 2009;**297**:R844–52

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