Trends in Cancer Research
Vol. 9, 2013

Review

Modulation of the opioid growth factor receptor alters the proliferation and progression of cancer

Nancy P. Kren, Ian S. Zagon and Patricia J. McLaughlin*

Department of Neural and Behavioral Sciences, Pennsylvania State University College of Medicine, Hershey, PA, 17033 USA

ABSTRACT

The opioid growth factor (OGF) - opioid growth factor receptor (OGFr) axis is present and tonically active in neoplastic as well as non-neoplastic cells. Addition of exogenous OGF decreases cancer cell growth *in vitro* and represses tumor growth in nude mice. In the presence of OGF the OGFr translocates to the nucleus and delays the cell cycle at the G1/S phase by upregulating p16 and/or p21 cyclin-dependent inhibitory kinases. Transient knockdown of OGFr gene and protein in 31 different human cancer cell lines resulted in altered growth characteristics suggesting that the OGF-OGFr axis is ubiquitous in human cancer. Transient knockdown of the classical opioid receptors did not affect growth characteristics, and OGF has been shown to be the specific ligand for OGFr. Genetic manipulation of human cancer cell lines to stably overexpress the nuclear associated receptor OGFr extended doubling times in culture, and decreased tumor incidence and size in nude mice xenografted with transfected human pancreatic cancer cells, squamous carcinoma of the head and neck cells, or ovarian cancer cells, relative to wildtype cancer cells. OGFr has been detected in a variety of human biopsy samples from normal tissue, as well as from benign or malignant tumors. Genetic and protein analyses of biopsied tumor specimens, and control tissues, from patients with squamous cell carcinoma of the head and neck or ovarian cancer revealed a loss of OGFr protein and binding activity with advanced stages of cancer. OGFr mRNA levels were comparable in advanced head and neck tumors relative to normal or benign tissues suggesting that the receptor is modified during the progression of cancer. In colorectal cancer as well as pancreatic cancer it was demonstrated that the receptor is unaltered with tumor progression. The alterations or modifications to OGFr are unknown and may be specific for each type of cancer. This article will review our knowledge about how the OGF-OGFr axis is modulated in human cancer cell proliferation and tumor progression.

KEYWORDS: OGFr, OGF, enkephalin, DNA synthesis, inhibitory growth factors, cancer, nude mice, *in vitro* studies

INTRODUCTION

The opioid growth factor receptor (OGFr) is a nuclear-associated opioid receptor that specifically binds the ligand [Met^5]-enkephalin for the regulation of cell proliferation during homeostasis and disease [1]. More than two decades of research on the identification, biochemical characterization, and immunocytochemical localization of the OGFr culminated in the subsequent cloning and sequencing of the gene for rat, mouse, and human, and determination of its location on chromosome 20q13.3 in humans [2-13]. OGFr is a 677 amino acid protein with binding sites of 32, 30, 17, and 16 kDa, determined by ligand binding in polyacrylamide denatured gels [13]; a fusion protein binding fragment of 62 kDa also has been isolated. Investigations in mouse neuroblastoma cells as well as in rat brain and cerebellum revealed that OGFr displays cellular

*Corresponding author: Pxm9@psu.edu

and tissue distribution profiles that distinguish it from other cytoplasmic membrane-bound, classical opioid receptors [2-6]. Although the receptor maintains some pharmacological characteristics similar to other receptors, OGFr bears no resemblance to classical opioid receptors (mu, delta, kappa; MOR, DOR, KOR) in amino acid sequence, structure or location [13-16]. MOR, DOR, and KOR are 7-transmembrane domain G-protein coupled receptors with their primary location being cytoplasmic membranes of neural cells [14-16].

OGFr distribution and structure

OGFr tissue distribution

The gene for OGFr and its protein have been identified in cells and tissues arising from all 3 dermal derivatives [10-12]. OGFr has been detected in prokaryotes [17] as well as in all vertebrate orders [7], and is prevalent in adult tissues that undergo constant replication [18-20]. Immunoreactive OGFr was localized to the cytoplasm of corneal epithelial cells in frog, turtle, bird, rodent, cat, non-human primate and man [7], as well as in normal colon [18], heart [19], and tongue [20] of rodents. Gene expression for OGFr has been documented in human fetal tissues including brain, liver, lung, and kidney as well as in adult heart, brain, liver, skeletal muscle, kidney, and pancreas [12]. OGFr mRNA was also detected in human placenta [12]. Binding assays on adult and fetal rat brain have quantitated OGFr binding [5, 6], and studies conducted in adult mice demonstrated RNA levels in brain, heart, lung, liver, kidney and skeletal muscle [12]. Additionally, OGFr has been localized in neoplasia, as well as in cell lines derived from human cancers [21-29].

Subcellular distribution of OGFr

Subcellular fractionation studies using developing rat brain and cerebellum revealed that OGFr binding is associated with the nuclear fraction [6]. Confocal microscopy studies in the rat cornea have demonstrated that OGFr and OGF are co-localized in the paranuclear cytoplasm as well as in the cell nuclei [8]. Immunoelectron microscopic studies demonstrated immunogold labeling of OGFr on the outer nuclear envelope, in the paranuclear cytoplasm and within the nucleus, adjacent to heterochromatin [8] in corneal epithelial cells. Co-localization of OGFr and OGF using differential sized immunogold particles and electron microscopy was evident on the outer nuclear envelope, in the paranuclear cytoplasm, as well as in the nucleus [8]. Collectively, these data indicate that the receptor is located on the outer nuclear envelope and translocates to the nucleus once bound to the endogenous ligand, OGF.

Structural characterization of OGFr

Unpublished observations suggest that OGFr is unstructured, however further studies are needed to confirm the NMR results. Comparison of amino acid similarities between mouse, rat, and human revealed 78% identity between mouse, rat, and human, and 87% similarity at the N terminus; C terminal homology dropped to 23% identity between human and rat, and 31% identity between mouse and rat [13]. Using COS-7 African green monkey kidney cells, as well as human cancer cell lines, the subcellular location of OGFr has been studied [30]. OGFr has three nuclear localization signals within its sequence, two mono-partite $NLS_{383-386}$ and $NLS_{456-460}$, and one bi-partite $NLS_{267-296}$ [30]. Studies utilizing site directed mutagenesis demonstrated that $NLS_{267-296}$ mutants had little to no effect on the distribution of the OGFr-EGFP fusion protein, while $NLS_{383-386}$ and $NLS_{456-460}$ mutations decreased nuclear localization by 40% and 30%, respectively. When $NLS_{383-386}$ and $NLS_{456-460}$ were both mutated the nuclear localization was decreased by 80%, indicating that $NLS_{383-386}$ and $NLS_{456-460}$ control nuclear localization while $NLS_{267-296}$ has little to no control [30]. When the $NLS_{383-386}$ and $NLS_{456-460}$ were both mutated, nuclear localization of OGFr was significantly decreased, and the regulatory effects of OGFr were diminished [30]. These observations indicate that the OGF-OGFr action on proliferation is dependent on the ability of OGFr to translocate into the nucleus [30]. As expected, the NLS(s) for OGFr is(are) recognized by karyopherin β and therefore its nuclear localization is dependent on the presence of karyopherin β as well as Ran protein [31].

Research studies at the Sanger Institute in Cambridgeshire, United Kingdom, involved sequencing human tumor samples as well as cancer cell lines and generating a “Catalogue of Somatic Mutations in Cancer” or COSMIC [32]. A total of 7718 samples across multiple tumor types were sequenced. Of these samples, 48 contain mutations within OGFr.

Thirty-four of the 48 mutations are missense or nonsense mutations with several of the same mutations occurring in multiple cancers. For example, a mutation termed S557T has been identified in prostate, skin and upper GI cancers [32]. Figure 1 demonstrates the specific mutations and their locations in reference to the three known NLS. Currently it is unclear if these mutations alter the function of the receptor and warrants further investigation.

Opioid growth factor (OGF) - the ligand for OGFr

The endogenous pentapeptide methionine enkephalin was identified in the 1970's [33-35] in brain and gut tissues in a scientific race to discover ligands associated with the newly identified opioid receptors. [Met^5]-enkephalin is a product of preproenkephalin and pro-opiomelanocortin genes and prohormones. OGF has been shown to be autocrine and paracrine, produced in cells and tissues, and is rapidly degraded upon secretion. Using a tissue culture model of human cancer cell lines (SCC-1, MIA PaCa-2), the focus of OGF action has been determined to be DNA synthesis. OGF targets DNA synthesis by interfering with the movement of cells through the G_1/S interphase of the cell cycle [36]. Further studies have shown that OGF coupled to OGFr translocates into the nucleus and alters cell cycle events. When OGF interacts with OGFr in the nucleus, p21 and p16 are upregulated and mediate a decrease in proliferation [37-39]. In normal non-neoplastic cells treatment with the exogenous ligand OGF induces increased expression of $p16^{INK4a}$ and $p21^{WAF1/CIP1}$, but has no effect on the expression of p15, p18, p19, or p27 [39]. Subsequent knockdown of p16 and p21 blocked the inhibitory effect of OGF, indicating that p16 and p21 are required for the OGF-OGFr axis to inhibit cell proliferation in normal cells [39]. Subsequent studies in human head and neck squamous cell carcinoma cell lines have demonstrated that the OGF-OGFr axis mediates cell growth through p16 [36]. Studies in human pancreatic cancer cell lines that lack p16 have reported that the OGF-OGFr axis is capable of working through p21 alone [37]. Thus, normal cells may require p21 as well as p16 to mediate the inhibitory effects of the OGF-OGFr axis, but it appears that these effects can be mediated through either p16 or p21 in cancer cell lines.

Function of the OGF-OGFr axis

In vitro studies

It is well established in multiple human cancers as well as in normal cell lines that exposure to exogenous OGF markedly decreases cell growth [21-29]. Zagon and colleagues demonstrated that the OGF-OGFr axis is present and tonically active in 31 diverse cancer lines, representative of roughly 90% of cancers that commonly affect humans [40], demonstrating that the OGF-OGFr axis is ubiquitously expressed across most, if not all, neoplasias, and can potentially be manipulated to provide therapy for a wide-range of cancers. Meticulous growth assays were conducted to assess the percent reduction in cell number following OGF treatment *in vitro*. Figure 2 is a representation of the collective reduction in growth (percent of control) recorded for multiple cell lines for each cancer.

Inhibitory action of OGF is specific and selective

Cell culture studies have demonstrated that OGF is the only opioid peptide that is capable of modulating proliferation. This was first demonstrated in a cell line of squamous cell carcinoma of the head and neck. CAL-27 cells that were treated with opioid and opioid-like compounds specific for MOR, DOR, and KOR at 10^{-6} M had no alterations in proliferation [24]. Similar results have been reported

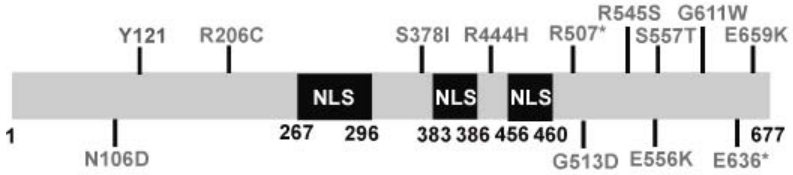

Figure 1. Diagram of the 34 missense mutations identified on OGFr in relationship to the three NLS, $NLS_{267-296}$, $NLS_{383-386}$, and $NLS_{456-460}$.

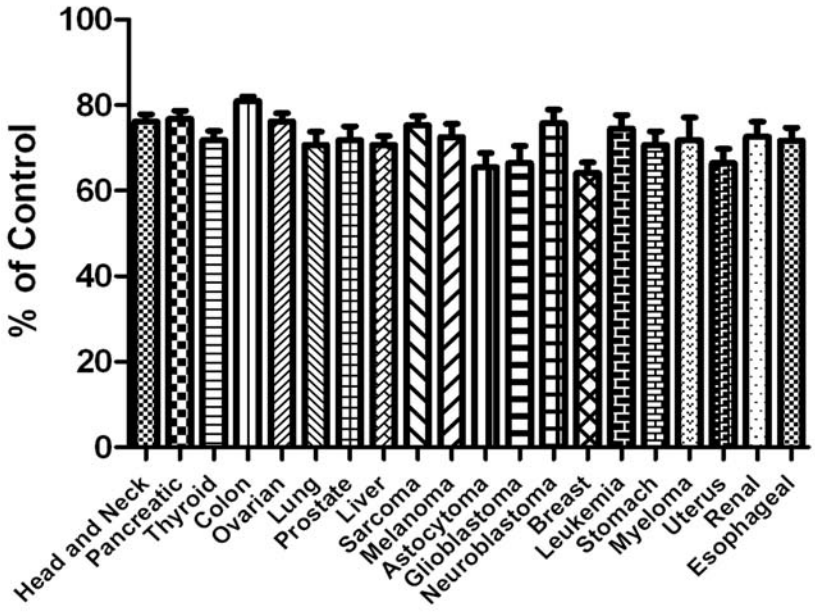

Figure 2. OGF inhibits cellular proliferation of a variety of cancers. Thirty-one human cancer cell lines were treated with OGF (10^{-6} M) for 72 hr. Cell number was counted using trypan blue exclusion methodology. Data (% of control) from all cell lines for each cancer are combined and expressed as means ± SEM. All values differed significantly from respective controls at $P < 0.05$. (Additional details provided in reference 40).

in tissue culture studies utilizing human neuroblastoma [23], thyroid follicular [25], ovarian [28], and breast cancer [29] cell lines. Studies with human pancreatic cell lines first reported that OGF was autocrine produced and secreted by cells [21], suggesting that endogenous OGF is active in inhibiting the proliferation of cells. The tonic regulation of cell growth has been confirmed in a variety of cell lines, and is evident by the significant increase in proliferation when cells in culture are treated with a polyclonal antibody specific for OGF. The inhibitory properties of exogenous OGF can also be mitigated by naloxone, a non-specific opioid receptor inhibitor. In cultures where OGF significantly decreases cell number, combination of naloxone and OGF negates the inhibitory effect of OGF, while naloxone alone has no effect on cell number [21]. Additionally, the tonic activity of endogenous OGF can be blocked by the opioid receptor antagonist naltrexone (NTX). Cells in culture exposed to NTX demonstrate increases in cell number [21]. The pharmacological principle of blockade by an opioid antagonist suggests that the effect on proliferation is mediated by an opioid receptor. Knockdown experiments using siRNAs conducted in the breast cancer cell line MDA-MB-231demonstrate that the inhibitory actions of OGF are mediated specifically by OGFr [29] (Figure 3). OGFr siRNA knockdown in cell culture resulted in a significant increase in cell number and negated the regulatory effects of OGF and NTX [29]. Additionally, knockdown of MOR, DOR or KOR had no effect on cell number and did not affect cellular response to OGF or NTX [29].

Modulation of OGFr alters the effectiveness of OGF

The regulatory effects of the OGF receptor can be investigated by increasing or decreasing the number of receptors in cancer cells. Overexpression of OGFr by expressing the pcDNA3.1+ OGFr-1 cDNA and establishing stable cell lines has been examined in pancreatic [41], head and neck [42], and ovarian cancer cell lines [43]. Overexpression of OGFr in the MIA PaCa-2 pancreatic cancer cell line results in a 47-91% increase in doubling times [41]. When OGFr was overexpressed in the head and neck cell line treatment of SCC-1 cells with OGF for 72 hr, it resulted in 200-550% greater decreases in cell number compared to wild-type and empty vector controls [42], suggesting that the overexpression of OGFr results in decreased proliferation and an increased response to exogenous OGF. In SCC-1

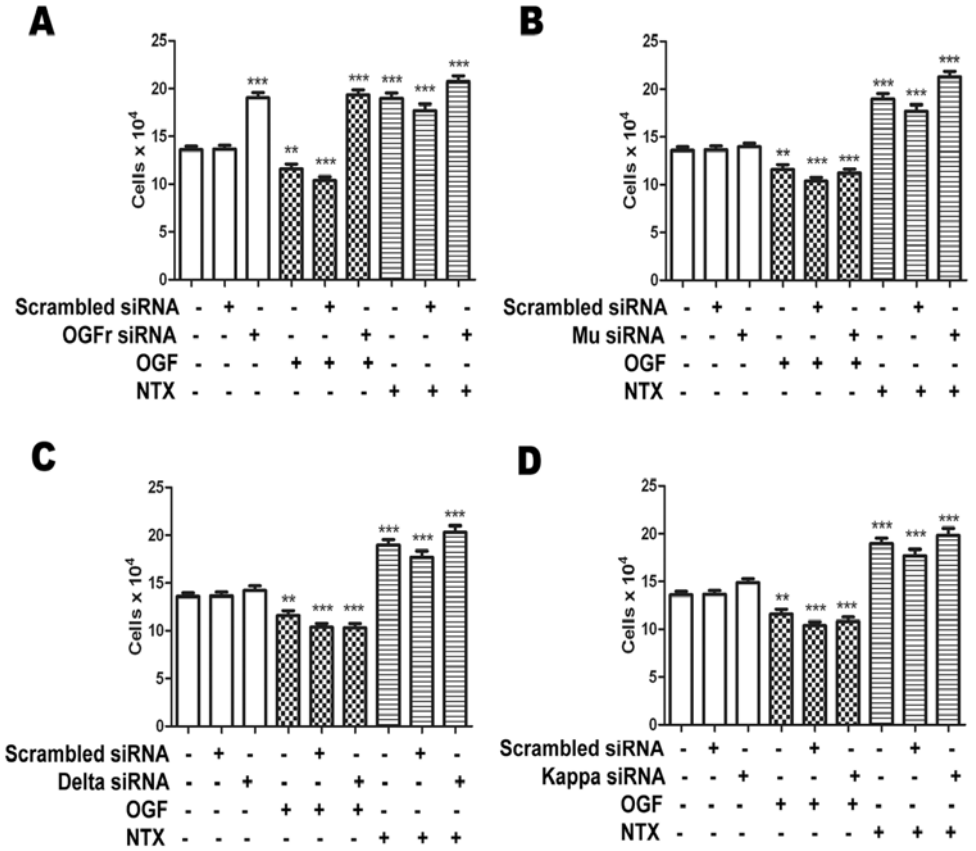

Figure 3. OGFr is required to mediate the inhibitory action of OGF in human triple negative breast cancer cell line MDA-MB-231. (A-D) Cells were transfected with (A) siRNA specific for OGFr, (B) siRNA specific for Mu opioid receptor, (C) siRNA specific for Delta opioid receptor and (D) siRNA specific for Kappa opioid receptor; untransfected or commercially available scrambled siRNA served as controls. Cells were treated with Opioid Growth Factor (OGF) (10^{-6} M), Naltrexone (NTX) (10^{-6} M) or untreated for 72 hr and counted. Data represent means ± SEM for at least 2 aliquots per well from 3 wells per group, and were analyzed using ANOVA with Newman-Keuls post hoc test. Significantly different from respective controls at **P < 0.01, ***P < 0.001. [Adapted from Zagon, I. S., Porterfield, N. K. and McLaughlin, P. J. 2013, Exp. Biol. Med., 238, 589, with permission from SAGE Publications, Inc].

cultures with overexpressed OGFr, depletion of endogenous OGF by polyclonal antibodies resulted in a two-fold increase in growth [42]. Similar results were seen in the ovarian cancer cell line SKOV-3, with a 36-158% decrease in cell number in transfected cells overexpressing OGFr [43]. In addition, studies in the ovarian cancer cell line SKOV-3 demonstrate that underexpression of OGFr could be achieved with shRNA constructs used to knockdown the endogenous expression of OGFr [43]. The cell number in each of the clonal lines characterized was increased by 32-132%, and doubling times were decreased by 29-35% compared to wild type and empty vector controls [43]. Additionally, cells underexpressing OGFr were no longer responsive to exposure to OGF and NTX [43]. It has also been demonstrated that partial blockade of OGFr with a low dose of NTX results in increased expression of OGFr and increased interaction between OGF and OGFr resulting in decreased proliferation [44]. Thus, *in vitro* studies have confirmed that the duration of OGFr blockade determines the direction of cell growth regulation such that cells exposed to NTX for 6 hr every 48 hr showed a 22-29% decrease in cell number, whereas cells exposed to NTX continuously showed a 22-42% increase in cell number [44]. Additionally, it was shown in the MDA-MB-231 cell line that

exposure to NTX for 4 hr every 24 hr had a similar effect to OGF, with 17.5-35% decrease in cell number [29]. OGFr can also be modulated by other drugs such as imiquimod [45]. Imiquimod is an imidazoquinoline compound that has been shown to modify immune response and has potent anti-viral and antitumor properties [46-49]. Utilizing pancreatic, colorectal, and squamous carcinoma of the head and neck cell lines, imiquimod upregulated the OGFr, which in turn, decreased cell proliferation [45]. The inhibitory effects of imiquimod were lost when OGFr was knocked down using siRNAs specific for OGFr [45]. Therefore, the anti-tumor properties of imiquimod may be mediated through the OGF-OGFr axis [45].

OGFr modulation in combination therapy

In addition to OGF's inhibitory properties when administered alone, OGF is also effective when combined with standard of care chemotherapy. *In vitro* studies examining the combination therapy of OGF and paclitaxel revealed reduced growth of 48-69% in the SCC-1 squamous cell carcinoma of the head and neck cell line relative to sterile-water control cultures [50]. Similar results were reported in the human pancreatic cancer cell line MIA PaCa-2. OGF in combination with gemcitabine or 5-fluorouracil markedly reduced cell number more than either agent administered in isolation [51] or with paclitaxel and ovarian cancer cells [52]. Investigations using the triple-negative breast cancer cell line MDA-MB-231, combinations of OGF (10^{-6} M) and paclitaxel (10^{-8} M) had results similar to that of paclitaxel (10^{-7} M) alone, but showed fewer dead cells in the combined therapy relative to paclitaxel alone [29]. These results indicate that OGF can work in combination with other pharmaceuticals, and may be able to mitigate some of the cytotoxic side effects of traditional chemotherapeutics [29]. Additionally it has been found that low dose NTX can also work in combination with standard of care therapeutics [44]. In the ovarian cancer cell line SKOV-3 it has been shown that paclitaxel or cisplatin in combination with OGF inhibits DNA synthesis more effectively than either compound alone [28]. Collectively, these results demonstrate that modulation of the OGF-OGFr axis in isolation and in combination therapies may be an important therapeutic option.

The OGF-OGFr axis and tumor progression

In vivo studies

Manipulation of the OGF-OGFr axis in an *in vivo* model was first demonstrated in 1983, when Zagon and colleagues demonstrated that a low dose (0.1 mg/kg) of NTX, termed LDN, resulted in a 33% decrease in tumor incidence when mice were inoculated with C1300 murine neuroblastoma. In addition to decreased tumor incidence, LDN delayed tumor appearance by 98% and increased survival by 36% relative to saline-treated tumor-bearing mice [53]. Interestingly, if mice were given intraperitoneal injections of a high dose of NTX (10 mg/kg), their tumors appeared earlier than controls (a 27% decrease in time), and the mice had a 19% reduction in survival time [53, 54]. Because NTX is a non-selective opioid antagonist, this drug blocks all opioid receptors. Therefore, treatment with LDN upregulates one or more opioid receptors. To determine which opioid receptor (MOR, DOR, KOR, OGFr) was responsible for the inhibitory effect of tumor growth, A/Jax mice were inoculated with S20Y murine neuroblastoma cell line and injected with multiple doses of OGF, ranging from 0.5-30 mg/kg, as well as other endogenous and exogenous ligands associated with classical opioid receptors [55]. Mice treated with Leu-enkephalin, D-Ala2, D-Leu-enkephalin, or ethylketocyclazocine exhibited little change in tumor progression. Only OGF increased latency and extended life span in a dose-related manner [55]. These data indicate that the inhibitory action of OGF is mediated through OGFr, since ligands selective for MOR, DOR and KOR had no effect. Similar paradigm-altering studies on the OGF-OGFr axis to validate the specificity and selectivity of ligand and receptor with regards to tumor progression have been repeated in nude mice inoculated with human cell lines for colon, pancreatic, squamous cell carcinoma of the head and neck (SCCHN), and ovarian cancers [56-66].

OGFr regulation of colon adenocarinoma

OGF inhibited the growth of colon cancer tumors generated by inoculating nude mice with the human colon cancer cell line HT-29 [56]. More than 80% of mice receiving OGF did not have visible tumors at 3 weeks post inoculation compared to 93% of control animals [56]. Animals treated with OGF

had a marked reduction in tumor size at all OGF concentrations [56]. The inhibitory effect of OGF was eliminated by treatment with naloxone [56]. Similar reductions in tumor size were observed with LDN therapy. Mice inoculated with HT-29 human colon cancer cells and receiving 0.1 mg/kg of NTX (LDN) daily displayed a 2.4-fold delay in tumor appearance relative to saline-treated tumor-bearing mice [57]. Plasma levels of OGF were elevated 2.5-fold in LDN-treated mice in comparison to control animals, indicating that the partial blockade of OGFr resulted in a significant increase in endogenous OGF [57].

OGFr regulation of pancreatic cancer

Nude mice subcutaneously inoculated with BxPC-3 human pancreatic adenocarcinoma cells were treated three times daily with OGF (5 mg/kg) or saline (controls) [58]. Animals treated with OGF had a 43% delay in the initial tumor appearance, and 63% of mice treated with OGF showed no signs of neoplasia at the time when tumor incidence was 100% for the saline-injected mice [58]. Mice injected with OGF had 24-fold greater levels of peptide in their tumor tissues than saline-treated animals; plasma levels in OGF injected mice were 8.6-fold lower than controls [58]. OGFr binding capacity, an indicator of receptor number, was reduced by 58% in tumor tissues from mice exposed to OGF [58] relative to controls. Additional studies on the human pancreatic adenocarcinoma cell line MIA PaCa-2 revealed that treatment with OGF (10 mg/kg) once daily significantly reduced tumor weight and tumor volume [61]. Animals exposed to OGF in combination with the standard of care chemotherapy drug gemcitabine (Gemzar) showed an additive inhibitory effect [61]. Combination of OGF (10 mg/kg) every day and Gemzar (120 mg/kg) given every 3rd day reduced tumor size by 70% from that recorded in mice subjected to OGF only, and 63% from subjects receiving only Gemzar [61]. Thus, pancreatic cancer can be reduced by altering the concentration of OGF, by treating with OGF or LDN alone, or by giving OGF in combination with chemotherapy drugs.

Modulation of the number of OGF receptors alters the progression of pancreatic cancer. Transfection of the MIA PaCa-2 cell line and subsequent growth analyses revealed that the stable cell lines expressed 3.3 times the amount of OGFr as was measured for cells transfected with empty vector or wild type cells. Tumor tissue generated from the OGFr overexpressing cell lines had 4.3 times more OGFr as tumors generated from empty vector and wild type lines [63]. Moreover, tumor incidence was reduced by up to 50%, latency was increased by 30%, and tumor volume was decreased by 70%, in mice inoculated with cells overexpressing OGFr in comparison with that recorded for animals receiving empty vector transfected cells or wildtype pancreatic cells [63].

Studies utilizing the human pancreatic cancer cell line Capan-2 revealed that OGFr is unaltered with tumor progression [62]. In this study immunohistochemistry as well as binding assays were used to characterize the amount of receptor present as well as its ability to bind to its ligand [62]. The size of the tumor, small, medium or large, did not have an effect on the number of OGF receptors present or the ability to bind ligand [62]. However, plasma levels of OGF were markedly decreased in mice with xenographed tumors compared to normal mice [62]. These studies indicate that OGF or LDN could be an alternative therapy for patients with pancreatic cancer.

OGFr regulation of squamous cell carcinoma of the head and neck

Nude mice inoculated with the SCCHN cell line, CAL-27 and treated with OGF had an 8-day increase in tumor latency and a reduction in tumor size of 16% relative to controls [59]. The inhibitory effect of OGF was blocked by treatment with naloxone. Mice treated with OGF and naloxone had tumors similar in size to those in the saline-treated group, indicating that the inhibitory actions of OGF are mediated by opioid receptors [59]. Animals treated either once or twice a day with OGF had no difference noted between the two treatment groups [59]. In addition to having an inhibitory effect on tumor growth, OGF was found to have similar effects in combination with the chemotherapy drug paclitaxel [65]. In this study the SCCHN cell line SCC-1 was inoculated in nude mice and the mice were treated with OGF (10 mg/kg) daily, paclitaxel (8 mg/kg) every other day or both OGF and paclitaxel [65]. On day 50 of the study, mice treated with OGF had a 33% decrease in tumor volume and paclitaxel/ OGF treated mice had a 69% decrease in tumor volume compared to controls [65]. Mice receiving

paclitaxel had reductions in tumor size, but also had marked decreases in body weight and life span, whereas mice treated with a combination of OGF and paclitaxel had similar body weights and life spans to control mice [65]. These data indicate that OGF moderates some of the toxic side effects of the standard of care chemotherapy drugs. In summary, the data suggest that modulation of the OGF-OGFr to inhibit proliferation of SCCHN is an important alternative therapy.

Moreover, the progression of SCCHN can be altered by overexpression of OGFr. Stable transfection of cDNA that overexpresses OGFr in SCC-1 cells resulted in new cell lines that had 2.5-3.7-fold more OGFr than wild type cells [66]. When the transfected cells were inoculated into nude mice the latency for tumor appearance was extended by 25-80%, and tumor size was decreased by 48-67% relative to controls [66]. The reductions in tumor size were attributed to the significant reduction in DNA synthesis, which was decreased by 45-65% in tumors generated by cell lines over expressing OGFr [66], supporting the hypothesis that overexpression of OGFr is an effective way to modulate SCCHN tumor progression. Other preclinical studies conducted in nude mice inoculated with three SCCHN cell lines (SCC-1, CAL-27, and SCC-15) demonstrated that the number of OGF receptors decreases with increased tumor size [64]. In this study it was shown that medium and large tumors had a 31-86% reduction in OGFr binding capacity compared to small tumors, and large SCCHN tumors had 26-79% less OGFr binding than medium tumors [64]. OGFr gene expression was comparable across the different sizes of tumor [64], suggesting that receptor number strongly correlates with tumor size, and presumably tumor progression, in SCCHN [64].

OGFr regulation of ovarian cancer

The OGF-OGFr axis has been extensively studied in human ovarian cancer cell lines grown *in vitro* or in nude mice, as well as patient tissue samples. The OGF-OGFr axis was observed to be present and functioning in human ovarian cancer. Donahue and colleges inoculated mice intraperitoneally with the ovarian cancer cell line SKOV-3 and treated the animals with OGF (10 mg/kg daily) or LDN (0.1 mg/kg daily) showing better outcomes for treated mice than saline treated animals [60, 67]. Mice injected with OGF or LDN displayed 42% and 39% decreases, respectively, in total number of tumors in the peritoneal cavity per mouse compared to controls [60, 67]. OGF or LDN reduced the cumulative tumor weight by 69% and 46% respectively [60, 67]. The reduction in tumor number and weight is associated with a decrease in cell proliferation, demonstrated by a significant reduction in DNA synthesis in mice from the OGF and LDN treatment groups [60, 67]. OGF or LDN were also effective in combination with standard of care chemotherapeutics such as cisplatin or paclitaxel [60, 67]. Mice treated with LDN and cisplatin had 26% decrease in tumor volumes and 25% decrease in tumor weight compared to mice treated with cisplatin alone [67]. In addition to the decreases in tumor weights and volumes, LDN appears to moderate some of the toxic side effects of cisplatin. Mice treated with cisplatin had significant weight loss in comparison to mice treated with cisplatin and LDN who had weights similar to those of saline-treated animals [67]. Tumors were also analyzed for DNA synthesis; animals treated with LDN and cisplatin had a 24% decrease in DNA synthesis compared to animals receiving cisplatin alone [67]. Additionally, the blood vessel density of tumors from mice treated with LDN and cisplatin was decreased by 43-44% compared to that of mice treated with cisplatin alone [67]. These data indicate that cisplatin and LDN can work in combination and may be an important alternative to standard of care treatments. The progression of ovarian cancer can also be modulated by over and under expression of OGFr. Mice were inoculated either subcutaneously or intraperitoneally with the ovarian cell line SKOV-3 engineered to overexpress OGFr [68]. SKOV-3 cell lines overexpressing the receptor with a significant overexpression (OGFr-3) or an intermediate overexpression (OGFr-22) were inoculated into nude mice [68]. At the termination of the study (day 32) 100% of wild type and empty vector controls had tumors, whereas only 40% of mice inoculated with OGFr-3 and 90% of mice inoculated with OGFr-22 expressed any visible sign of tumors [68], demonstrating that in some cases, tumor growth was completely prevented by molecular modulation of OGF receptor number. Mice inoculated with the OGFr-3 cell line

had a 12-day increase in latency for tumor appearance, while the mice receiving OGFr-22 transfected cells showed a 4-day increase in latency for tumor appearance relative to animals receiving wildtype or empty-vector treated cell lines [68]. Thus, the progression of ovarian cancer can be modulated by an overexpression of OGFr, and the overall effect of the overexpression is dose dependent. DNA synthesis in the tumors was also significantly decreased by overexpression of OGFr; OGFr-3 tumors displayed a 78% decrease and OGFr-22 tumors had a 67% decrease in DNA synthesis [68]. Analyses of blood vessel density in the tumors indicated that the overexpression of OGFr mediates the progression of ovarian cancer by decreasing cell division and angiogenesis [68].

Ovarian cancer cell lines were molecularly engineered to underexpress OGFr [69]. The SKOV-3 cell line was transfected with shRNA against OGFr and stable lines were generated that had significant reductions in OGFr expression, with up to 53% decreases from wild type cells [69]. To asses the progression of ovarian cancer in the presence of decreased OGFr, mice inoculated with cell lines under expressing OGFr demonstrated 100% tumor incidence as early as day 7, while wild type and empty vector groups did not have 100% tumor incidence until days 19 and 20, respectively [69]. These data indicated that a loss of OGFr amplifies ovarian tumor growth [69]. In addition to having decreased tumor latency, mice inoculated with cells under expressing OGFr had accelerated tumor growth; however, the tumors reached their optimal tumor size by day 4 [69]. Not unexpectedly, treatment with OGF did not alter growth kinetics in tumors generated from under expressing lines [69].

Clinical

The OGF-OGFr axis is a novel biological pathway that can be manipulated to inhibit cancer cell proliferation and tumor progression. Investigations using human tissues for assessment of the OGF-OGFr axis are limited [70-74] and only a few human cancer clinical trials using OGF [73, 74] have been completed. It appears that the presence and stability of OGF ligand and receptor, OGFr, are critical for regulation of cancer cell replication. OGF given exogenously, or upregulated by low dosages of naltrexone, are new, non-toxic, and safe therapies for cancer patients. As biotherapies, these ligands can be added to other chemotherapeutic regimens for additional efficacy, and may in fact, confer some protection against the toxicity of known therapeutics. The receptor plays a critical role. Little is currently known about how the function of the receptor is regulated. Biopsied tissue samples from benign ovarian cysts or malignant ovarian tumors revealed marked reductions in OGFr expression [72]. In SCCHN, OGFr protein levels decreased significantly with tumor progression, but OGFr mRNA levels were comparable [71]. These observations suggest that the number of receptors available may be altered during post-translational processing, potentially by modifications such as phosphorylation, methylation or ubiquitination. Several large scale phosphoproteome studies have identified several residues of OGFr as phosphorylated [75-85]. Additionally, some of these residues are phosphorylated exclusively in the cytoplasm [76] or their phosphorylation state changes with the progression of the cell cycle [79] indicating that these phosphorylations could be regulating the function of the receptor based on location or cell cycle. The number of receptors could also be regulated by degradation. In a proteome-wide study looking at ubiquitinylation, two residues of OGFr were identified as ubiquitinated [86]; additionally, in large protein stability studies the half-life of OGFr was shown to be sensitive to the proteasome inhibitor MG 132 [87]. Predictive software such as MeMo also indicates that OGFr may be methylated [88]. Additional molecular studies are needed to ascertain the regulatory effect of each of these post-translational modifications. Molecular changes in OGFr that render it unable to bind OGF, or in low supply, could have a significant effect on the progression of cancer as well as affecting the response to OGF biotherapy.

ACKNOWLEDGEMENTS

The primary research discussed in this review was supported by grants from NIH, Philip Morris USA, PA Department of Health, and the Shockey Family Foundation. The authors express their gratitude to the numerous technicians and students who conducted much of the work.

CONFLICT OF INTEREST STATEMENT

NPK has no conflict of interest. ISZ and PJM are listed as inventors on patents assigned to Penn State University for use of OGF as a therapy for cancer. This intellectual property was licensed to TNI Biotech (Bethesda, MD). ISZ and PJM are listed on patents assigned to the inventors on OGFr.

REFERENCES

1. McLaughlin, P. J. and Zagon, I. S. 2012, Biochemical Pharmacol., 84, 746.
2. Zagon, I. S., Goodman, S. R. and McLaughlin, P. J. 1988, Brain Res., 449, 80.
3. Zagon, I. S., Goodman, S. R. and McLaughlin, P. J. 1990, Brain Res., 511, 181.
4. Zagon, I. S., Gibo, D. M. and McLaughlin, P. J. 1991, Brain Res., 551, 28.
5. Zagon, I. S., Gibo, D. M. and McLaughlin, P. J. 1992, Brain Res., 596, 149.
6. Zagon, I. S., Goodman, S. R. and McLaughlin, P. J. 1993, Brain Res., 605, 50.
7. Zagon, I. S., Sassani, J. W., Allison, G. M. and McLaughlin, P. J. 1995, Brain Res., 671, 105.
8. Zagon, I. S., Ruth, T. B., Leure-duPree, A. E., Sassani, J. W. and McLaughlin, P. J. 2003, Brain Res., 967, 37.
9. Zagon, I. S., Ruth, R. B. and McLaughlin, P. J. 2005, Anat Rec., 282A, 24.
10. Zagon, I. S., Verderame, M. F., Allen, S. and McLaughlin, P. J. 1999, Brain Res., 849, 147.
11. Zagon, I. S., Verderame, M. F., Zimmer, W. E. and McLaughlin, P. J. 2000, Molec. Brain Res., 84, 106.
12. Zagon, I. S., Verderame, M. F., Allen, S. and McLaughlin, P. J. 2000, Brain Res., 856, 75.
13. Zagon, I. S., Verderame, M. F. and McLaughlin, P. J. 2002, Brain Res. Rev., 38, 351.
14. Pert, C. B. and Snyder, S. H. 1973, Science, 179, 1011.
15. Terenius, L. 1973, Acta Pharmacol. Toxicol. (Copenh), 32, 317.
16. Simon, E. J., Hiller, J. M. and Edelman, I. 1973, Proc. Natl. Acad. Sci. USA, 70, 1947.
17. Zagon, I. S. and McLaughlin, P. J. 1992, Life Sci., 50, 1179-1187.
18. Zagon, I. S., Wu, Y. and McLaughlin, P. J. 1997, Am. J. Physiol., 272, R1094.
19. Wu, Y., Zagon, I. S. and McLaughlin, P. J. 1998, Dev. Dyn., 211, 327.
20. Zagon, I. S., Wu, Y. and McLaughlin, P. J. 1994, Amer. J. Physiol., 267, R645.
21. Zagon, I. S., Smith, J. P. and McLaughlin, P. J. 1999, Int. J. Oncol., 14, 577.
22. Zagon, I. S., Hytrek, S. D. and McLaughlin, P. J. 1996, Amer. J. Physiol., 271, R511.
23. McLaughlin, P. J., Zagon, I. S. and Skitzki, J. 1999, Int. J. Oncol., 14, 373.
24. McLaughlin, P. J., Levin, R. J. and Zagon, I. S. 1999, Int. J. Oncol., 14, 991.
25. McLaughlin, P. J., Goldenberg, D., Park, S. S., Conway, A., Donahue, R. N. and Zagon, I. S. 2009, BMC Cancer, 9, 369.
26. Bisignani, G. J., McLaughlin, P. J., Ordille, S. D., Jarowenko, M. J. and Zagon, I. S. 1999, J. Urol., 62, 2186.
27. Avella, D. M., Kimchi, E. T., Donahue, R. N., Tagaram, H. R., McLaughlin, P. J., Zagon, I. S. and Staveley-O'Carroll, K. F. 2010, Amer. J. Physiol. Regulatory Integrative Comp. Physiol., 298, R459.
28. Donahue, R. N., McLaughlin, P. J. and Zagon, I. S. 2009, Amer. J. Physiol., 296, R1716.
29. Zagon, I. S., Porterfield, N. K. and McLaughlin, P. J. 2013, Exp. Biol. Med., 238, 589.
30. Cheng, F., McLaughlin, P. J., Verderame, M. F. and Zagon, I. S. 2009, Exp. Biol. Med., 234, 532.
31. Cheng, F., McLaughlin, P. J. and Zagon, I. S. 2010, Exp. Biol. Med., 235, 1093.
32. Forbes, S. A., Bindal, N., Bamfor, S., Cole, C., Kok, C. Y., Beare, D., Jia, M., Shepherd, R., Leung, K., Menzies, A., Teague, J. W., Campbell, P. J., Stratton, M. R. and Futreal, P. A. 2011, Nucleic Acids Research, 39, D945.
33. Simon, E. J., Hiller, J. M. and Edelman, I. 1973, Proc. Natl. Acad. Sci. USA, 70, 1947.
34. Terenius, L. 1973, Acta Pharmacol. Toxicol., 33, 377.
35. Hughes, J., Smith, T. W., Kosterlitz, H. W., Fothergill, L. H., Morgan, B. A. and Morris, H. R. 1975, Nature, 258, 577.
36. Zagon, I. S., Roesener, C. D., Verderame, M. F., Ohlsson-Wilhelm, B. M., Levin, R. J. and McLaughlin, P. J. 2000, Int. J. Oncol., 17, 1053.
37. Cheng, F., Zagon, I. S., Verderame, M. F. and McLaughlin, P. J. 2007, Cancer Res., 67, 10511.
38. Cheng, F., McLaughlin, P. J., Verderame, M. F. and Zagon, I. S. 2008, Mol. Cancer, 7, 5.

39. Cheng, F., McLaughlin, P. J., Verderame, M. F. and Zagon, I. S. 2009, Mol. Biol. Cell, 20, 319.
40. Zagon, I. S., Donahue, R. N. and McLaughlin, P. J. 2009, Amer. J. Physiol., 297, R1154.
41. Zagon, I. S., Verderame, M. F., Hankins, J. L. and McLaughlin, P. J. 2007, Int. J. Oncol., 30, 775.
42. McLaughlin, P. J., Verderame, M. F., Hankins, J. L. and Zagon, I. S. 2007, Int. J. Mol. Med., 17, 421.
43. Donahue, R. N., McLaughlin, P. J. and Zagon, I. S. 2011, J. Cancer Therapy, 4, 579.
44. Donahue, R. N., McLaughlin, P. J. and Zagon, I. S. 2011, Exp. Biol. Med., 236, 1036.
45. Zagon, I. S., Donahue, R. N., Rogosnitzky, M. and McLaughlin, P. J. 2009, Exp. Biol. Med., 233, 968.
46. Stanley, M. A. 2002, Clin. Exp. Dermatol., 27, 571.
47. Sauder, D. N. 2003, Brit. J. Dermatol., 149, 5.
48. Oldfield, V., Keating, G. M. and Perry, C. M. 2005, Amer. J. Clin. Dermatol., 6, 195.
49. Miller, R. L., Gerster, J. F., Owens, M. L., Slade, H. B. and Tomai, M. A. 1999, Int. J. Immunopharmacol., 21, 1.
50. McLaughlin, P. J., Jaglowski, J. R., Verderame, M. F., Stack, B. C., Leure-duPree, A. E. and Zagon, I. S. 2005, Int. J. Oncol., 26, 809.
51. Zagon, I. S., Jaglowski, J. R., Verderame, M. F., Leure-duPree, A. E., Smith, J. P. and McLaughlin, P. J. 2005, Cancer Chemother Pharmacol., 56, 510.
52. Donahue, R. N., Zagon, I. S. and McLaughlin, P. J. 2011, J. Cancer Ther., 2, 110.
53. Zagon, I. S. and McLaughlin, P. J. 1983, Science, 221, 671.
54. Zagon, I. S. and McLaughlin, P. J. 1984, Life Sci., 35, 409.
55. Zagon, I. S. and McLaughlin, P. J. 1988, Life Sci., 43, 1313.
56. Zagon, I. S., Hytrek, S. D., Lang, C. M., Smith, J. P., McGarrity, T. J., Wu, T. and McLaughlin, P. J. 1996, Am. J. Physiol., 271, R780.
57. Hytrek, S. D., McLaughlin, P. J., Lang, C. M. and Zagon, I. S. 1996, Cancer Letters, 101, 159.
58. Zagon, I. S., Hytrek, S. D., Smith, J. P. and McLaughlin, P. J. 1997, Cancer Letters, 112, 167.
59. McLaughlin, P. J., Levin, R. J. and Zagon, I. S. 2003, Cancer Letters, 199, 209.
60. Donahue, R. N., McLaughlin, P. J. and Zagon, I. S. 2011, Gynecol. Oncol., 122, 382.
61. Zagon, I. S., Jaglowski, J. R., Verderame, M. F., Leure-duPree, A. E., Smith, J. P. and McLaughlin, P. J. 2005, Cancer Chemother. Pharm., 56, 510.
62. Zagon, I. S. and McLaughlin, P. J. 2006, Int. J. Oncol., 29, 489.
63. Zagon, I. S., Kreiner, S., Heslop, J. J., Conway, A. B., Morgan, C. R. and McLaughlin, P. J. 2008, Int. J. Oncol., 33, 317.
64. McLaughlin, P. J. and Zagon, I. S. 2006, Int. J. Oncol., 28, 1577.
65. Jaglowski, J. R., Zagon, I. S., Stack, B. C., Verderame, M. F., Leure-duPree, A. E., Manning, J. D. and McLaughlin, P. J. 2005, Cancer Chemother. Pharmacol., 56, 97.
66. McLaughlin, P. J., Kreiner, S., Morgan, C. R. and Zagon, I. S. 2008, Int. J. Oncol., 33, 751.
67. Donahue, R. N., McLaughlin, P. J. and Zagon, I. S. 2011, Exp. Biol. Med., 236, 883.
68. Donahue, R. N., McLaughlin, P. J. and Zagon, I. S. 2011, J. Cancer Ther., 2, 579.
69. Donahue, R. N., McLaughlin, P. J. and Zagon, I. S. 2012, Exp. Biol. Med., 237, 167.
70. Hytrek, S. D., Smith, J. P., McGarrity, T. J., McLaughlin, P. J., Lang, C. M. and Zagon, I. S. 1996, Amer. J. Physiol., 271, R115.
71. McLaughlin, P. J., Stack Jr., B. C., Levin, R. J., Fedok, F. and Zagon, I. S. 2003, Cancer, 97, 1701.
72. Fanning, J., Hossler, C. A., Kesterson, J. P., Donahue, R. N., McLaughlin, P. J. and Zagon, I. S. 2012, Gynecol. Oncol., 124, 319.
73. Smith, J. P., Ahmad, M., Conter, R., Bingaman, S., Harvey, H., Mauger, D., Demers, L., McLaughlin, P. J., Stanley, W. and Zagon, I. S. 2004, Anticancer Drugs, 15, 203.
74. Smith, J. P., Bingaman, S. I., Mauger, D. T., Harvey, H. H., Demers, L. M. and Zagon, I. S. 2010, Open Access J. Clin. Trials, 2, 7.
75. Gnad, F., Gunawardena, J. and Mann, M. 2010, Nucleic Acids Research, 39, D253.
76. Olsen, J. V., Blagoev, B., Gnad, F., Macek, B., Kumar, C., Mortensen, P. and Mann, M. 2006, Cell, 127, 635-648.
77. Matsuoka, S., Ballif, B. A., Smogorzewska, A., McDonald, E. R. III., Hurov, K. E., Luo, J., Bakalarski, C. E., Zhao, X., Solimini, N., Lerenthal, Y., Shiloh, Y., Gygi, S. P. and Elledge, S. J. 2007, Science, 316, 1160.

78. Dephoure, N., Zhou, C., Villen, J., Beausoleil, S. A., Bakalarski, C. E., Elledge, S. J. and Gygi, S. P. 2008, PNAS, 105, 10767.
79. Olsen, J. V., Vermeulen, M., Santamaria, A., Kumar, C., Miller, M. L., Jensen, L. J., Gnad, F., Cox, J., Jensen, T. S., Nigg, E. A., Brunak, S. and Mann, M. 2010, Science Signaling, 3, ra3.
80. Oppermann, F. S., Gnad, F., Olsen, J. V., Hornberger, R., Greff, Z., Keri, G., Mann, M. and Duab, H. 2009, Molecular & Cellular Proteomics, 8(7), 1751.
81. Mayya, V., Lundgren, D. H., Hwang, S., Rezaul, K., Wu, L., Eng, J. K., Rodionov, V. and Han, D. K. 2009, Science Signaling, 84, ra46.
82. Gauci, S., Helbig, A. O., Slijper, M., Krijgsveld, J., Heck, A. J. R. and Mohammed, S. 2009, Analytical Chemistry, 81, 4493.
83. Han, G., Ye, M., Zhou, H., Jiang, X., Feng, S., Jiang, X., Tian, R., Wan, D., Zou, H. and Gu, J. 2008, Proteomics, 8, 1364.
84. Zanivan, S., Gnad, F., Wickstrom, S. A., Geiger, T., Macek, B., Cox, J., Fassler, R. and Mann, M. 2008, Journal of Proteome Research, 7, 5314.
85. Wisniewski, J. R., Nagaraj, N., Zougman, A., Gnad, F. and Mann, M. 2010, Journal of Proteome Research, 9, 3280.
86. Wagner, S. A., Beli, P., Weiner, B. T., Nielsen, M. L., Cox, J., Mann, M. and Choudhary, C. 2011, Molecular & Cellular Proteomics, 10, M111.013284.