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Protein Modification and Functional Implications for Oct-4 in Human Stem Cells

ISHTIAQ AHMAD, DANIEL C. HOESSLI, EVELYNE WALKER-NASIR, AHMAD KHURSHID, SALEEM M. RAFIK, ABDUL RAUF SHAKOORI AND NASIR-UD-DIN*

Institute of Molecular Sciences and Bioinformatics, Lahore, Pakistan (IA, EWN, AK, SMR, NUD), Department of Pathology and Immunology, CMU, University of Geneva, Geneva, Switzerland (DCH), ARP C & S, Geneva, Switzerland(SMR) and School of Biological Sciences, University the Punjab, Lahore, Pakistan (ARS).

Abstract:- A number of nuclear and cytosolic proteins have been identified that are modified by certain dynamic post-translational modifications by addition of *O*-GlcNAc (O-link N-acetylglucoseamine) and phosphate. These modifications play an important role in modulating their functions. Majority of transcriptional factors have been found to be modified by *O*-GlcNAc and these modifications are transitory with phosphate. Octamer binding protein-4 (Oct-4), an embryonic stem cell marker, was originally identified as a DNA-binding protein that activates gene transcription via a *cis*-element containing octamer motif. It is one of the POU transcription factors. Its expression has been observed in totipotent embryonic stem and germ cells. A critical level of Oct-4 expression is required to sustain stem cell self-renewal and pluripotency. Differentiation of embryonic stem cells results in down- regulation of Oct-4, an event essential for a proper and divergent developmental program. It is being proposed that post-translational modifications including *O*-GlcNAc addition and phosphate addition along with their interplay play a vital role in multiple switches of silencing of certain genes and activation of other genes in same or different cells resulting in specific differentiation of a cell.

Key words: Oct-4**,** protein modification, protein function, human stem cells.

INTRODUCTION

In mammalian embryogenesis the first morphological indication of differentiation is the formation of the trophoectoderm (TE) at the early blastocyst stage. While inner cell mass (ICM) remains totipotent, TE cells are restricted to extra-embryonic cell lineages. Data from various animal models suggest that, on a molecular level, this differentiation step could start in humans perhaps as early as the 2–4-cell stage and might involve the activation of maternally inherited determinants and/or embryo-specific genes, as well as environmental factors such as cell allocation (Edwards and Beard, 1997). In humans, an understanding of the molecular mechanisms underlying this differentiation step may provide further exploratory and diagnostic means. Currently, blastomere biopsies of human embryos are performed for preimplantation genetic diagnosis (PGD) around the 8-cell stage and no prediction can be made regarding the future fate of these cells. However, in some instances combined knowledge about ploidy and destination towards ICM or TE may be crucial and critical in selecting the best embryos for transfer.

In mice, Oct-4 is exclusively found in totipotent embryonic cells and germ cells (Palmieri *et al*., 1994). A high level of Oct-4 expression is considered to keep cells in a totipotent stage, whereas down-regulation is associated with differentiation (Palmieri *et al*., 1994); Oct-4 transcription occurs prior to any changes in known transcription factor levels (Brehm *et al*., 1997). In 8-cell stage murine embryos, five cells stain immunohistochemically positive for Oct-4 protein and three negative (Palmieri *et al*., 1994). After day 8 of murine embryonic development Oct-4 is restricted to primordial germ cells. It is also expressed in murine embryonic stem (ES) cells (Rosner *et al*., 1990) and embryonal carcinoma (EC) cells (Okamoto *et al*., 1990).

In humans, Oct-4 is the product of the *OTF3* gene, which encodes two splicing variants

*To whom correspondence be addressed.
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designated *Oct3A* and *Oct3B* (Takeda *et al*., 1992). Human *Oct-4* (*oct3a*) shares 87% sequence identity with mouse *Oct-4* (Takeda *et al*., 1992). In human embryos, *Oct-4* is expressed throughout from the unfertilized oocyte to the blastocyst stages, as detected by reverse transcription–polymerase chain reaction (RT–PCR) (Abdel-Rahman *et al*., 1995) and by PCR of cDNA libraries (Verlinsky *et al*., 1998). *Oct-4* is also expressed in human EC (Pera and Herszfeld, 1998) and ES cells (Reubinoff *et al*., 2000). Recently it has been reported that *Oct-4* is upregulated by ~31-fold in the ICM of human blastocysts compared with TE cells, making it a possible tool to produce and maintain human ES cells (Hansis *et al*., 2000).

Transcriptional regulators can exhibit tissue-specific expression, and they can be sorted into related families on the basis of conserved amino acid sequences of their DNA-binding domains (Mitchell and Tjian, 1989). Oct-4 and FoxD3 (previously Genesis) are two transcriptional regulators whose expression is highly limited to ES cells. Oct-4 is a member of the POU homeodomain family of transcriptional regulators also known to be critical in embryonic development (Pesce and Scholer, 2001). Oct-4 is expressed almost exclusive in ES cells before implantation, and is down-regulated at the blastocyst stage and gastrulation, where somatic lineages are first defined at that stage (Hromas *et al.*, 1999). The level of Oct-4 expression drives the decision of the blastocyst to form ultimately either embryonic or extraembryonic tissue (Pesce and Scholer, 2001; Niwa *et al*., 2000). Specifically, high levels of Oct-4 designate cells to become extra-embryonic mesoderm or endoderm such as the yolk sac, normal Oct-4 levels make ES cell totipotent, and low levels designate cells to become tropho-ectoderm, such as the placenta (Niwa *et al*., 2000).

Most transcription factors appear to be *O*-GlcNAcylated by *O*-Glucosaminyltransferase (OGT) in the transcriptional activation domains (Comer and Hart, 1999), and Sp1 is no exception (Jackson and Tjian, 1988), suggesting that OGT plays a critical role in the control of protein-protein interactions involved in transcriptional activation. Using an *in vitro* transcription system, *O*-GlcNA modification of the SpE peptide markedly inhibited its ability to activate transcription. The first transcription factor shown to bear the *O*-GlcNAc modification was Sp1 (Jackson and Tjian, 1988), a ubiquitous transcription factor that plays a vital role in the control of TATA-less housekeeping gene transcription (Pugh and Tjian, 1991).

Several lines of evidence suggest that phosphorylation of octamer factors may provide a mechanism of differential regulation of octamer factor binding to high- and low-affinity binding sites (Kapiloff *et al*., 1991; Grenfell *et al*., 1996).

The octamer motif is a regulatory DNA element involved in both ubiquitous and cell-type-specific gene expression and is recognized by a family of eukaryotic transcription factors that share a homologous bipartite DNA binding domain, the POU domain (Sturm and Herr, 1988; Verrijzer and van der Vliet, 1993; Herr and Cleary, 1995). The POU domain consists of two structurally independent sub-domains: a 75-amino-acid amino-terminal region specific for this class of transcription factors (POUS) and a 60-amino-acid carboxy-terminal homeodomain (POUH). Both sub-domains make specific contacts with DNA through a helix-turn-helix structure (Klemm *et al*., 1994) and are connected by a variable linker that can vary from 15 to 56 amino acids in length.

A number of octamer-binding proteins are expressed in distinct tissue-specific patterns and are believed to mediate cell-type-specific gene regulation during development, perhaps in combination with additional factors that can interact either with the POU domain or with activation domains (Schöler *et al.*, 1991; Herr and Cleary, 1995). Several lines of evidence suggest that phosphorylation of octamer factors may provide a mechanism of differential regulation of octamer factor binding to high- and low-affinity binding sites (Kapiloff *et al*., 1991; Grenfell *et al*., 1996). Moreover, the difference in C-domain activity correlates with a difference in phosphorylation status of the Oct-4 protein in different cell lines that also suggests a mechanism by which C-domain function may be regulated (Brehm *et al*., 1997).

The dynamic and regulatory *O*-GlcNAc modification sites were found to be similar to the phosphorylation sites (Comer and Hart, 1999; Wells *et al*., 2001; Kamemura *et al*., 2002). An interplay

between glycosylation and phosphorylation has been noted in nucleoplasmic and cytoplasmic proteins (Comer and Hart, 1999; Wells *et al*., 2001; Kamemura *et al*., 2002). *O*-GlcNAc modification in transcription factors has been shown to be involved in modulating the function of these proteins (Yang *et al*., 2002). We propose here the involvement of *O*-GlcNAc modification along with its interplay with phosphorylation in modulating the function of Oct-4 differently in same and different cells.

MATERIALS AND METHODS

Sequence data

The sequence of the Oct-4 (oct-3/4) has been described (Wey *et al*., 1994; Takeda *et al*., 1992) since nineties. The sequence data used for predicting phosphorylation and glycosylation sites for oct-3/4 of human was retrieved from SWISSPROT sequence database with entry name PO51_HUMAN and primary accession number Q01860. The sequence of human Oct-4 was also retrieved from Entrez (NCBI sequence viewer) that was found to be identical to SWISSPROT entry. So no difference was found with respect to number and sequence position of amino acids between the sequences retrieved from the two sources.

Prediction methods

Prediction methods used to predict potential glycosylation sites involved four methods; three for potential *O*-linked glycosylation sites and one for predicting *N*-linked glycosylation sites. Methods used for predicting potential O-linked glycosylation sites include *NetOGlyc* 3.1 (http://www.cbs.dtu.dk/services/NetOGlyc/), that predicts O-glycosylation sites in mucin type proteins (i.e., for O-GalNAc sites), *DictyOGlyc* 1.1 (http://www.cbs.dtu.dk/services/DictyOGlyc/) predicts *O-GlcNAc* sites in eukaryotic proteins and YinOYang 1.2 (http://www.cbs.dtu.dk/services/YinOYang/), that predicts O-GlcNAc sites in eukaryotic proteins. The *NetNGlyc* 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) was used for predicting N-glycosylation sites. The above-mentioned four methods for predicting the glycosylation sites are neural network based. For prediction of phosphorylation sites in selectins *NetPhos 2.0* (http://www.cbs.dtu.dk/services/NetPhos/) was used. The *NetPhos* 2.0 is also a neural network based program designed by training the neural networks through protein phosphorylation data from phosphobase 2.0.

RESULTS

Prediction results of Oct-4 for different *O*-linked and *N*-linked glycosylation sites show that the protein has the potential for both *N*-linked and *O*-linked glycosylation. Among *O*-linked glycosylation sites α-GalNAc and β-GlcNAc are the most frequent ones. The prediction results for *O*-β-GlcNAc modification show that there are a total of seven potential sites highly predicted to be modified by *O*-GlcNAc at Ser 288, 306, 311, 349, and 359 and at Thr 116 and 351 (Fig. 1). Apart from this there are total 08 other residues (Ser 55, 105, 109, 289, 335 and 355; Thr 118 and 331) which are predicted to be non-glycosylated but very close to the threshold and have a chance to be modified by *O*-GlcNAc. Among these 08 residues 04 are also predicted as positive sites for phosphorylation by NetPhos 2.0, so are likely to be the possible interplay sites (Fig. 1).

Similarly, the prediction results by Netphos 2.0 for possible phosphorylation sites show that Oct-4 possesses a high potential for phosphate modification. A total of 21 residues are predicted to be phosphorylated (Fig. 2). These 21 residues include 14 Ser (at 12, 55, 93, 105, 107, 111, 193, 236, 288, 289, 290, 303, 327 and 336), 3 Thr (at 118, 183 and 235), and 4 Tyr (at 67, 292, 325 and 334). The high ratio of Ser to Tyr residues for predicted phosphate modification suggests possible high *O*-GlcNAc modification in Oct-4.

DISCUSSION

Nucleocytoplasmic *O*-GlcNAc modification is dynamic and abundant, exhibiting characteristics more like those of phosphorylation than those of typical *N*- and *O*-linked glycosylation (Chou *et al*., 1992; Chou and Omary, 1993). There may be more than 2000 protein kinases encoded in the mammalian genome, each with its own subset of substrates, the OGT gene appears to be a single copy gene in metazoan genomes (Shafi *et al*., 2000).

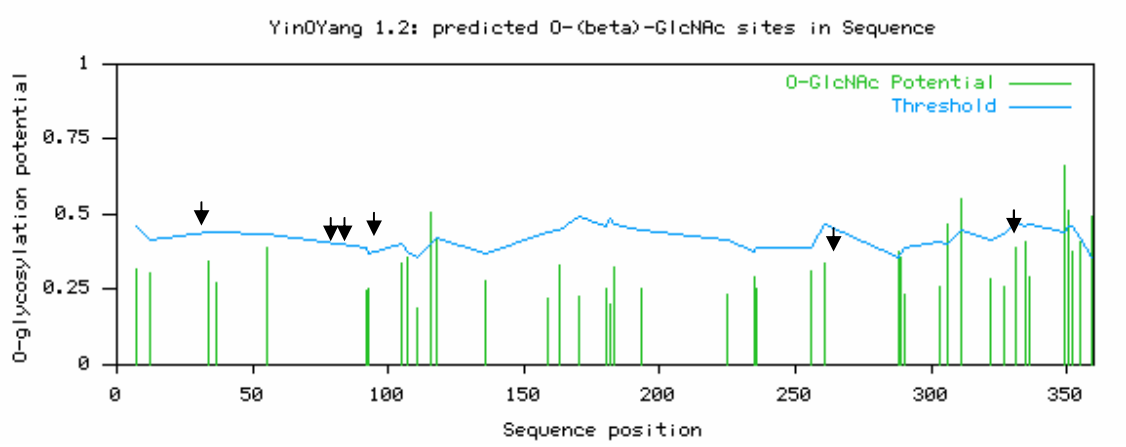

Fig. 1. Graphical presentation of potential sites for *O*-GlcNAc modification at Ser and Thr. Vertical lines crossing threshold line show potential to be modified by *O*-GlcNAc but the vertical lines very close to threshold are also likely to be modified by *O*-GlcNAc represented by arrow heads.

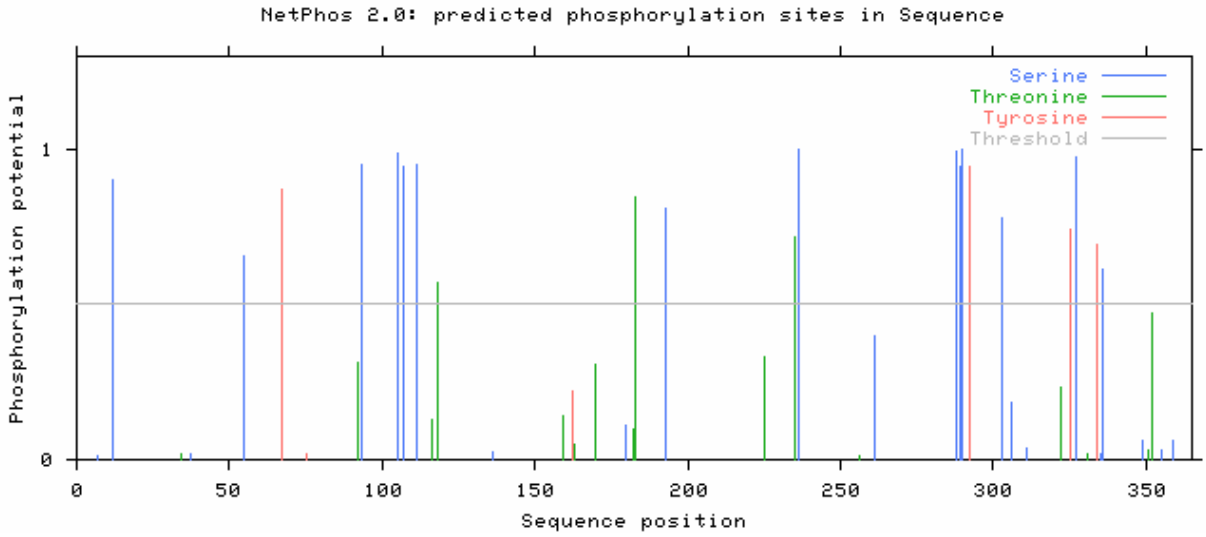

Fig. 2. Graph showing the potential sites for phosphate addition at Ser, Thr, and Tyr with a fix threshold of 0.5.

However, OGT protein catalyzes *O*-GlcNAc modification of numerous transcription factors and other intracellular proteins, implying that this enzyme displays much greater flexibility in recognizing its many substrates (Wells *et al*., 2001) than do the protein kinases. One hallmark of the OGT molecule is its tandem TPR motifs, which are very conserved throughout evolution and exist in a wide range of proteins (Lamb *et al*., 1995; Roos and Hanover, 2000). Distinct regions in the OGT TPR array might selectively contact a diverse set of transcription factors and modulate their glycosylation states after being attracted to promoters by a corepressor (Yang *et al*., 2002). OGT may exhibit specific conformations. This property may contribute to the ability of OGT to modify a diverse range of protein substrates with no discernable motif (Yang *et al*., 2002) by *O*-GlcNAc.

Furthermore, the involvement of OGT in transcriptional repression was also proposed (Yang *et al*., 2002).

In more than one cases the site of *O*-GlcNAc modification is the same as phosphorylation. For example, c-myc is *O*-GlcNAc-modified at Thr 58, a known site of phosphorylation and a mutational hot spot in lymphomas (Chou *et al*., 1995). Recent work suggests that glycosylation of c-myc plays a role in protein stability and subcellular localization of the transcription factor (Kamemura *et al*., 2002). The Sp-1 transcription factor is one of the most well studied *O*-GlcNAc-modified proteins. Work has shown that *O*-GlcNAc modification of Sp-1 appears to modulate its transactivation capability (Roos *et al*., 1997). Interestingly OGT activity was shown to be required for maximal transcriptional repression. Furthermore, increased *O*-GlcNAc modification of p53 and NF-UL are associated with increased transcriptional activity (Vosseller *et al*., 2002). Thus, there is convincing data showing that *O*-GlcNAc modification of the transcriptional machinery is modulating activity. It remains to be elucidated how *O*-GlcNAc is modulating activity at the molecular level and how *O*-GlcNAc modification of the transcription machinery is itself being regulated.

An interesting example of a transcription factor with an activation domain that is negatively regulated by its DNA binding domain is ATF-2 (Li and Green, 1996; Livingstone *et al*., 1995). Similarly, an amino-terminal region of ATF-2 displays transactivation function when fused to the DNA binding domain of Gal4 (Gal4DBD) but is silent in the context of the native protein. The basic helix-loop-helix DNA binding domain of ATF-2 is required to inhibit activation domain function and directly interacts with the cryptic activation domain *in vitro*. ATF-2 function is stimulated by stress-activated protein kinases belonging to the mitogen-activated protein kinase family which phosphorylate two threonine residues within the activation domain. This phosphorylation has been proposed to disrupt an inhibitory interaction between the activation domain and the DNA binding domain as being observed for Oct-4, the inhibition of the activation domain is cell line specific.

Brehm *et al*. (1997) have shown that the ability of the C domain to function as a transactivation domain correlates with differential post-translational modifications of the Oct-4 protein in the different cell lines. Phosphorylation on Oct-3 *in vivo* has been found exclusively on Ser residues but not required for its binding to DNA. But addition of *O*-GlcNAc to corepressor like mSin3A can block the activity of transcriptional activator resulting in gene silencing (Yang *et al.,* 2002).

In multiple *in vitro* systems, Oct-4 down-regulation is essential for mammalian ES cells to differentiate to defined lineages (Pesce and Scholer, 2001; Niwa *et al*., 2000). Although only a few targets of Oct-4 transcriptional regulation are known (Pesce and Scholer, 2001; Niwa *et al*., 2000), Oct-4 can function as a homo- or heterodimer on palindromic octamer DNA sequences (classic consensus ATTTGCAT) to repress or activate transcription according to flanking sequence or chromatin structure (Pesce and Scholer, 2001). For example, Oct-4 activates expression of fibroblast growth factor-4 and osteopontin in ES cells, but represses the ß-human chorionic gonadotropin promoter in ES cells (Pesce and Scholer, 2001). Oct-4 may require interacting factors such as Sox-2 or E1A to mediate transcriptional activation (Pesce and Scholer, 2001). Both Oct-4 and FoxD3 expression is down-regulated by the differentiating agent retinoic acid, which tends to drive undifferentiated cells to express neural markers (Pesce and Scholer, 2001; Niwa *et al*., 2000).

From the above discussion it is clear that *O*-GlcNAc modification of transcriptional factors contribute importantly in their functioning. We propose that predicted potential of O-GlcNAc modification sites (that lie both in C-terminal and N-terminal domains) may contribute significantly even by transitory modifications to glycosylation and phosphorylation, and thereby modulate the Oct-4 function. Phosphorylation in Oct-3 is not required for its binding (Yang *et al.,* 2002). But *O*-GlcNAc, on the basis of its similarity to sp1, can also result in changing of its binding activity. So Ser 55, 105, 107 and Ser 289, 306, 311, 349 and 359 on its termini along with DNA binding domain at Ser 288, 289 can play crucial role in modulating the multiple switches of Oct-4 during differentiation by silencing some genes and activating the others. Moreover, it is

quite probable that phosphorylation at the same residues that are potential sites for *O*-GlcNAc can block specific residues, thus allowing other residues to be modified by *O*-GlcNAc resulting in activation and repression of specific genes in same or different cells destining them to differentiate differently.

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