THE ROLE OF O-GLCNAC TRANSFERASE IN ZEBRAFISH EMBRYOGENESIS

by

DANIELLE M. WEBSTER

(Under the Direction of Scott Dougan)

ABSTRACT

The study of development addresses the question of how an organism is formed from a single cell. Cells communicate with each other to instruct the fate of their neighboring cells, induce cell death, cell migration and morphogenesis. Processes such as the induction of cell fate are controlled by secreted factors such as activin, BMP and Nodal signals. Signal transduction pathways transmit many of the secreted factors involved in the process of development. Typical signal transduction is mediated by modifying proteins with chemical groups, such as phosphorus that may change the activity of the cytoplasmic or nuclear proteins (Gilbert, 2000). This results in gene transcription or repression, which leads to changes in cell fate. Once cell fates are specified they organize themselves by migrating during a process known as morphogenesis. Cell migration and morphogenesis are controlled by other factors such as the cytoskeleton and cell-cell adhesion. To understand development it is important to understand how modifications alter protein activity. O-linked glycosylation is another common posttranslational modification found to be involved in development, however its role is not fully understood. Regulation of O-GlcNAc occurs in the nucleus and cytoplasm and modifies many proteins such as transcription factors, oncogenes and cytoskeletal proteins. O-GlcNAc Tranferase (OGT) is the enzyme that catalyzes the addition of O-GlcNAc. OGT controls various reactions potentially affecting the activity of many proteins involved in signal transduction. Much is known about the proteins it modifies by in vitro biochemical and genetic studies, but little is known about how the modification affects protein activity in vivo.

We are examining the role of OGT during zebrafish embryogenesis. Unlike mammals, zebrafish have two ogt genes, which encode several isoforms of the protein. Embryos overexpressing or lacking the enzyme are slowed during epiboly and have dramatically altered morphology at 24hpf. Several factors involved in zebrafish epiboly such as E-cadherin and cytoskeletal proteins have been shown to be modified by OGT in cell culture. We have examined the role of OGT and its effects on these factors that affect epiboly. We found that OGT affects cell adhesion between blastomeres along the cytoskeleton.

INDEX WORDS: Morphogenesis, glycosylation O-GlcNAc, O-GlcNAc Transferase, O-GlcNAcase, E-cadherin, epiboly
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DEDICATION

This dissertation is dedicated to my loving grandmother Gloria M. Box, who taught me to scorn delights and live laborious days, my supportive parents Sadie and Peter Webster who have made countless sacrifices for me to achieve all my goals, and my very best friends who have supported me during my time in graduate school who always pushed me to never give up.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>ACKNOWLEDGEMENTS</th>
<th>v</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td><strong>CHAPTER</strong></td>
<td></td>
</tr>
<tr>
<td>1 INTRODUCTION AND REVIEW OF LITERATURE</td>
<td>1</td>
</tr>
<tr>
<td>2 O-LINKED GLYCOSYLATION REGULATES ENODODERM FORMATION AND MORPHOGENESIS IN ZEBRAFISH EMBRYOS</td>
<td>33</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>34</td>
</tr>
<tr>
<td>BACKGROUND</td>
<td>35</td>
</tr>
<tr>
<td>RESULTS</td>
<td>37</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>46</td>
</tr>
<tr>
<td>CONCLUSION</td>
<td>48</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>49</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>51</td>
</tr>
<tr>
<td>3 CONCLUSION</td>
<td>74</td>
</tr>
<tr>
<td>APPENDICES</td>
<td>79</td>
</tr>
<tr>
<td>A THE POSTTRANSLATIONAL MODIFICATION O-GLCNAC PLAYS A ROLE DURING VERTEBRATE DEVELOPMENT</td>
<td>79</td>
</tr>
<tr>
<td>B THE ROLE OF V.2 ZEBRAFISH O-GLCNAC TRANSFERASE IN EMBRYOGENESIS</td>
<td>109</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Schematic of a zebrafish embryo</td>
<td>26</td>
</tr>
<tr>
<td>1.2</td>
<td>Map of the three presumptive germ layers of the zebrafish</td>
<td>27</td>
</tr>
<tr>
<td>1.3</td>
<td>The cytoskeleton of the zebrafish embryo</td>
<td>28</td>
</tr>
<tr>
<td>1.4</td>
<td>The zebrafish embryo</td>
<td>29</td>
</tr>
<tr>
<td>1.5</td>
<td>Diagram of Nodal signaling pathway and BMP signaling pathway</td>
<td>30</td>
</tr>
<tr>
<td>1.6</td>
<td>Structure of O-GlcNac Transferase (OGT) and O-GlcNAcase</td>
<td>31</td>
</tr>
<tr>
<td>1.7</td>
<td>OGT and O-GlcNAcase are responsible for the addition and removal of the O- GlcNAc modification</td>
<td>32</td>
</tr>
<tr>
<td>2.1</td>
<td>Expression pattern of zebrafish OGT</td>
<td>59</td>
</tr>
<tr>
<td>2.2</td>
<td>Depleting OGT causes morphological defects and a reduction in endodermal precursor cells</td>
<td>61</td>
</tr>
<tr>
<td>2.3</td>
<td>Manipulating levels of O-GlcNAc does not affect early specification</td>
<td>63</td>
</tr>
<tr>
<td>2.4</td>
<td>Overexpressing zOGT causes similar defects as depleting levels of zOGT</td>
<td>65</td>
</tr>
<tr>
<td>2.5</td>
<td>Overexpressing zOGT does not affect brain specification but causes a reduction in endoderm</td>
<td>67</td>
</tr>
<tr>
<td>2.6</td>
<td>Overexpressing zOGT results in defects during gastrulation</td>
<td>69</td>
</tr>
<tr>
<td>2.7</td>
<td>Overexpressing zOGT causes a delay in epiboly</td>
<td>71</td>
</tr>
<tr>
<td>2.8</td>
<td>Overexpressing zOGT disrupts the cytoskeleton</td>
<td>73</td>
</tr>
</tbody>
</table>
Figure A.1: Overexpressing the enzyme responsible for O-GlcNAc causes morphological defects.................................................................98

Figure A.2: hgg1 expression is altered when OGT is overexpressed .........................100

Figure A.3: Mesoderm is still specified when OGT is overexpressed .........................102

Figure A.4: Expressing rNOGT causes morphological defects similar to expressing rOGT.....104

Figure A.5: Manipulating levels of O-GlcNAc causes defects during gastrulation ........106

Figure A.6: Altering levels of O-GlcNAc leads to a reduction of endoderm, but not ectoderm 108

Figure B.1: Identifying zebrafish OGT ........................................................................126

Figure B.2: Manipulating levels of O-GlcNAc causes morphological defects ..............128

Figure B.3: Overexpressing OGT causes a reduction in endoderm .............................130

Figure B.4: OGT plays a role during morphogenesis ................................................132

Figure B.5: Defects during gastrulation are caused by a delay in epiboly ......................134

Figure B.6: Overexpressing v.2 zOGT causes a decrease in cell-cell adhesion ..............136
CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

In eukaryotic cells, many nuclear and cytoplasmic proteins are modified by the O-linked addition of β-N-acetylglucosamine (O-GlcNAc) to serine and threonine residues. This post-translational modification is highly dynamic, but significant questions remain about how this modification regulates protein activity and what is the effect of this modification on whole animals. Unlike phosphorylation, which is catalyzed by dozens of kinases, a single enzyme, called O-GlcNAc Transferase (OGT) catalyzes the addition of O-GlcNAc to a wide variety of target proteins. Removal of O-GlcNAc is catalyzed by O-GlcNAcase. In Arabidopsis, OGT mutants affect the ability of cells to respond to the growth hormone, gibberellin. Thus in plants, OGlcnAcylation regulates a specific developmental signalling pathway. In mice, however, OGT knock-outs are embryonic stem cell lethal, which has made it difficult to study the role of this enzyme during mammalian development. In zebrafish, there are two OGT genes, each of which encodes six different isoforms of the enzyme that are expressed maternally and zygotically. We demonstrate that OGT is required for zebrafish embryogenesis. Increasing OGT levels by mRNA injection inhibits endoderm formation and causes a severe delay in epiboly. The resulting embryos have a dramatically altered morphology at 24hpf. Reducing OGT function with a translation blocking anti-sense morpholino oligonucleotide causes similar defects, suggesting that cycling of O-GlcNAc is essential. The defects in OGT expressing embryos superficially resemble those in embryos lacking the maternal and zygotic contributions of
Spg/Oct4. This suggests that O-GlcNAc regulates Oct4 function. We have also examined whether the epiboly defects in OGT expressing embryos can be attributed to defects in the actin or microtubule-based cytoskeleton. We found that embryos with increased levels of OGT have disorganized yolk microtubules, which are required for epiboly. In addition, the actin cytoskeleton is reduced in the blastomeres, and expanded in the extra-embryonic yolk syncytial layer. This suggests that a defect in the adherens junctions. Consistent with this, cells occasionally fall off the blastoderm, indicating severe defects in cell adhesion. Our data is the first analysis of vertebrate embryos with increased or decreased levels of OGT. We have established a role for OGT during morphogenesis and, possibly, regulating Spg/Oct4 function.

**Zebrfish Development**

In a zebrafish embryo the cells undergo rapid cleavage and develop on top of an extra-embryonic structure known as the yolk cell (Figure 1.1; Kimmel et al., 1995). At 3 hours post fertilization (hpf) the YSL is formed when the blastomeres in direct contact with the yolk collapse and release their cytoplasm and nuclei into the adjacent yolk cell. The blastoderm keeps in contact with the yolk via the yolk syncytial layer (YSL). The YSL is comprised of the internal (I-YSL) that is characterized by loosely packed nuclei, and the external (E-YSL), which is characterized by densely packed nuclei (Kimmel, 1995). The remaining portion of the yolk i
known as the YCL or the yolk cytoplasmic layer (Figure 1.1). This is the non-nucleated portion of the yolk cell. The YSL is one of the first structures to send signals to the blastomeres to instruct cell fate decisions and cell migration, however little is known about all the factors that drives these processes.

Gastrulation is one of many morphogenic events that occur during embryogenesis and leads to the formation of the three germ layers - the ectoderm, mesoderm and endoderm (Figure 1.2). There are three processes that drive the establishment of the proper embryonic structure. These are epiboly, involution and convergence and extension.

*Zebrafish Epiboly*

Just before gastrulation the embryo is organized with the presumptive germ layers along the animal-vegetal axis (Figure 1.2). At about four hours post fertilization (hpf) the cells of the blastula flatten and migrate around the yolk in the process called epiboly. Epiboly is the first to occur of the three cellular movements that shape the embryo during gastrulation. Together the blastomeres and YSL simultaneously spread to cover the yolk during epiboly. Studies in *Fundulus* show that the YSL progresses through epiboly even after the removal of the blastoderm cap, supporting the evidence for the YSL involvement in zebrafish epiboly (Betchaku and Trinkaus, 1978). Cells move between one another along an axis in a process called radial intercalation. This is responsible for driving cells to move from the interior of the embryo to a more superficial position forcing a spreading of the cell around the yolk, thereby driving epiboly (Rhode and Heisenberg, 2007). Once cells of the blastoderm have undergone radial intercalation
they adapt a flattened shape, which is also thought to be another driving force during epiboly (Rohde and Heisenberg 2007).

The cells of the blastoderm are composed of an outer layer of surface cells known as the enveloping layer (EVL) and the underlying deep cells (DC). During epiboly the underlying deep cells undergoing radial intercalation do not enter the EVL (Warga and Kimmel, 1990). Also, cells of the EVL do not undergo involution and convergence (Warga and Kimmel, 1990). The EVL seems to play a passive role in the rearrangement of the cells of the blastoderm, however it is pulled down over the yolk cell by the yolk syncytial layer (YSL).

*The role of E-cadherin in zebrafish epiboly*

There are three molecular processes that drive epiboly (Rohde and Heisenberg 2007). Firstly, there is regulation of cell-cell adhesion within the blastomeres by E-cadherin (Solnica-Krezel and Cooper, 2002). Cadherins are membrane proteins involved in cell-cell adhesion (Takeichi 1991). They contain an N terminal extracellular domain, which usually promotes homotypic interactions with partners with neighboring cells. There is also a transmembrane anchor that is linked to a C terminal intracellular domain. The C terminal domain of cadherins interacts with α, β, and γ catenins, which then link the cadherin to the actin cytoskeleton (Potukka and Weis, 2007). E-cadherin was the first member of the cadherin family to be discovered and plays a role in the adhesion of epithelial cells (Takeichi, 1988). E-cadherin was first identified in the embryo as a result of its involvement with the mouse morula when antibodies against E-cadherin inhibited compaction in the early mouse embryo (Hyafil et al.,
1980; Takeichi, 1988). Since then it has been implicated in many developmental processes such as early morphogenesis in the zebrafish (Kane and Adams, 2002; Babb and Marrs, 2004).

There are other members of the cadherin family such as N-cadherin and P-cadherin that are also involved in the formation of junctions (Sacco et al., 1995; Yonemura et al., 1995). E-cadherin acts in complex with β and γ-catenin, along with the intracellular actin filaments to promote cell-cell adhesion.

In zebrafish E-cadherin (cdh1) is expressed maternally ubiquitously. It is also required for adhesion of the blastomeres (Babb et al., 2001). Depleting E-cadherin by translation blocking morpholino oligonucleotides in the zebrafish leads to defects in cell adhesion, as the blastomeres do compact on top of the yolk leading to cells shedding from the embryo (Babb and Mars, 2004). E-cadherin is not only involved in cell-cell adhesion, but also plays a role later in development during epiboly and gastrulation processes that require fine modulation of cell adhesiveness. This establishes its involvement in zebrafish morphogenesis (Babb and Mars, 2004).

Haploid screening of zebrafish ENU-induced mutants isolated a morphogenic movement mutant (Shimizu et. al, 2005). The hypomorphic mutation mapped to the rk3 locus that encodes for zebrafish E-cadherin (cdh1rk3) (Shimizu et. al., 2005). Maternal zygotic mutants of cdh1rk3 show embryos with an arrest in epiboly similar to embryos with depleted E-cadherin. This indicated E-cadherins involvement in zebrafish epiboly (Shimizu et. al., 2005). These cdh1rk3 mutants also showed significant similarities to the zygotic recessive half baked mutants (hab). *hab* mutants embryos which also encodes the zebrafish homolog of E-cadherin have defects in epibloy (Shimizu et al., 2005; Kane et al., 2004). In zebrafish E-cadherin mutants the blastoderm
does not undergo proper radial intercalation and embryos have significant epiboly defects (Kane et al 1996a, 2005). E-cadherin’s requirement in epiboly is apparent due to mutants of e-cadherin displaying epiboly defects during gastrulation.

*Microtubule involvement in zebrafish epiboly*

Another major driving force during epiboly results from microtubules within the YSL and the yolk cell. Solnica-Krezel and Driever demonstrated that there are two arrays of microtubules extending from the YSL in zebrafish. The first is an intercrossing network within the YSL, while the second derives from the network in the YSL and extends along the animal vegetal axis (Figure 1.3; Solnica-Krezel and Driever, 1994). Ultraviolet irradiation of the microtubule network that extends along the animal-vegetal pole from the YSL resulted in defects in epiboly indicating the involvement of microtubules in zebrafish epiboly (Stahle and Jesuthasan, 1993). Disruption of these microtubules using Taxol, a microtubule stabilizing agent, also resulted in a delay in epiboly (Solnica-Krezel and Driever, 1994). It was also shown that microtubule disruption during gastrulation leads to epiboly defects within the three germ layers (Cheng et al., 2004). When blastomeres are removed from the embryo the YSL still undergoes epiboly movement to cover the yolk. This indicates that the yolk microtubules are essential for epiboly rather than the microtubules of the blastomeres (Solnica –Krezel and Driever, 1994).
Actin cytoskeletal structures in zebrafish epiboly

Microtubules are not the only cytoskeletal structures within the cytoskeleton that play a role in driving epiboly. Betchaku and Trinkaus found that the thin microfilaments within the YSL of Fundulus pull on the cells of the attached EVL during epiboly (Betchaku and Trinkaus, 1978). This was the first indication of a role for microfilaments for the rearrangement of cells during epiboly. Initial studies in zebrafish embryos treated with the microtubule depolymerizing agent, nocodazole, resulted in disruption of epiboly. However, there was not complete disruption of the movements of the EVL and DC as their vegetal expansion were not inhibited but only slowed (Solnica-Krezel and Driever, 1994). This suggested that microtubules could not be the only source driving epiboly. The presence of thin and thick microfilaments within the cortex and cytoplasm of the EVL and YSL in Fundulus had been shown previously to be involved in the process of epiboly (Betchaku and Trinkaus, 1978). F-actin localizes in the blastomeres of zebrafish embryos during the first stages of epiboly (Zalik et al., 1999). F-actin also colocalizes with cell surface E-cadherin for the formation of cell junctions. Marginal cells of the blastomeres however, remain F-actin rich, but no E-cadherin is present within these cells (Zalik et al., 1999). This suggested a role for F-actin in early epiboly. Later studies showed actin-based structures to be localized in YSL zebrafish even after 50% epiboly (Cheng et al., 2004). The presence of a ring of actin within the YSL promotes contraction of the YSL resulting in EVL epiboly (Figure 1.3; Koppen et. al., 2006). Knock down of the transcription factor mtx2 causes a loss of the F-actin ring, which results in a delay in zebrafish epiboly (Wilkins et al., 2007). These results support the role of F-actin in zebrafish epiboly.
Involution

The formation of the epiblast (a more superficial layer) and hypoblast (the inner cells) is known as a process called involution. At about 50% epiboly, about 5 hpf the blastoderm begins to organize into three cellular layers. Presumptive mesoderm and endoderm cells of the blastoderm begin to internalize (Rohde and Heisenberg, 2007). Once inside these cells continue to migrate towards the animal pole and form the internal layer or the hypoblast. The non-internalizing cells, the ectoderm, continue epiboly and form the epiblast (Kimmel, 1990). This is the second process of cellular movements that occurs in shaping the embryos (Warga et. al., 1990).

Convergence and Extension

Convergence and extension allows for cells to accumulate on the dorsal side of the embryo establishing and lengthening the embryonic axis (Warga et. al., 1990). This results in elongation of the axis and changes the shape of the embryo from a ball to a rod. The dorsal accumulation of cells is known as convergence, while extension refers to the elongation of the anterior–posterior axis (Rohde et. al., 2007). This process begins about the same time as involution and is driven by cell intercalation and directed migration (Rohde and Heisenberg, 2007; Myers et al., 2002). The onset of convergence and extension is noted by the formation of the zebrafish organizer or embryonic shield, which is visualized an accumulation and thickening of cells on the dorsal side of the embryo (Fig1.4; Warga and Nusslein-Volhard, 1998). Convergence and extension occur to narrow the germ layers and elongate the embryo from head
to tail. Epiboly, involution, convergence and extension each occur to orient the cells and tissues of an embryo allowing for the establishment of a well-patterned organism.

**Zebrafish Cell Fate**

Fate mapping shows which area of an embryo is destined to develop into specific tissues (Figure 1.4). Labeling dyes are injected into the earlier embryo and used to observe an undifferentiated cell’s movement with their correlating developmental fate (Kimmel et al. 1990). Mesoderm and endoderm are derived from cells located closest to the margin where the cells meet the yolk (Figure 1.2; Kimmel et al., 1995). Mesoderm extends further towards the animal pole than endoderm and ectoderm is located in the most animal regions (Figure 1.2). Derivatives of mesoderm include notochord, somites, heart, blood and pronephros (Kimmel, Ballard et al., 1995). Derivatives of endoderm include stomach, pancreas, intestines and liver (Figure 1.4). A cell’s fate is determined by signals given by its neighboring cells (Kimmel et al., 1990). These signals come from signaling centers in the embryo known either as the YSL or a dorsal structure known as the organizer. The YSL forms about 3 hours post fertilization and cues from the YSL are responsible for patterning the animal vegetal axis. The zebrafish organizer known as the shield, forms at about 6 hours post fertilization (Figure 1.4). Signals from the organizer are responsible for patterning the dorsal-ventral axis of the zebrafish. Prechordal plate, notochord and brain are derived from the dorsal axis (Figure 1.4; Kimmel et al., 1995). Skin and blood are derived from ventrally located cells (Figure 1.4; Kimmel et al., 1995).
At 24 hours a zebrafish embryo is patterned (Kimmel et al., 1995). The notochord runs along the body axis, the eye is anterior and the heart beats under the eye (Figure 1.4).

The role of Nodal Signaling in zebrafish development

The establishment of the embryonic axis to induce the formation of the three germ layers, the mesoderm, endoderm and ventral neural ectoderm are controlled by Nodal signals. Nodal related proteins are a subset of the TGFβ superfamily of secreted proteins (Schier, 2003).

First discovered in the mouse, Nodal genes were found to play a significant role in mammalian gastrulation as nodal mutant mouse lack the primitive streak and were deficient in mesodermal derivatives. (Zhou et al., 1993; Conlon et al., 1991, 1994). Mouse, human and chick embryos have a single nodal related gene, however, zebrafish have three, squint (sqt), cyclops (cyc) and southpaw (spaw) (Conlon et al 1994; Feldman et al., 1998; Long et al., 2003; Fan and Dougan, 2007). The nodal-related genes sqt and cyc are also required for formation of the germ-layers in the zebrafish as sqt;cyc mutants are deficient in head and trunk mesoderm and lack all derivatives of endoderm (Feldman et al., 1998; Sampath et al., 1998). The third nodal related gene spaw is involved in regulation of left-right asymmetry and is expressed after gastrulation (Long et al., 2003). Depletion and overexpression of nodal-related genes establish their requirement for the germ layers and body axis for all vertebrate development.
Nodals act in a concentration dependent manner to pattern the animal-vegetal axis (Chen and Schier, 2001). The Nodal ligand binds to and activate the type II receptor ActRIIB through phosphorylation on ser/thr residues by a kinase (Reissmann, Jornvall et al. 2001). The phosphorylated type II receptor then binds to the type I receptor Alk4 (Massague 1998). The activated type I receptor phosphorylates and activates SMAD 2 and/or SMAD 3 (Reissman et al., 2001; Chen et al., 1996; Roijer et al., 1997). Phosphorylated SMAD 2/3 form a complex with SMAD 4 and translocates to the nucleus to activate downstream genes (Figure 1.5; Shioda, Lechleider et al., 1998).

**BMPs are responsible for patterning the dorsal-ventral axis**

Pre-gastrula embryos are also patterned along the dorsal ventral axis (Kimmel et al., 1995). Ventral fates are controlled by bone morphogenic proteins (BMPs), which are members of the TGF-β superfamily (Hammerschmidt et al., 1996; Serbedzija et al., 1996). The transcription factor β-catenin induces the expression of noggin and chordin, which antagonize BMP localizing BMP ventrally in the embryo (Schulte-Merker et al 1997). Noggin and chordin bind to BMP in the extracellular matrix and prevent them from binding their receptors (Schulte-Merker et al., 1997). BMP acts through a signal transduction pathway via SMADS that is similar to the nodal related pathway. However, BMP binds to receptors that phosphorylate and activate SMAD 1 and SMAD5. Phosphorylated SMAD1/5 forms a complex with SMAD 4 (Kretzschmar et al., 1997; Doody et al., 1997; Kretzschmar et al., 1997a; Liu et al. 1997). SMADS get translocated to the nucleus for transcription of downstream genes (Figure 1.5).
The role of O-GlcNAc Transferase in early zebrafish development

The structure of O-GlcNAc Transferase (OGT)

O-GlcNAc Transferase (OGT) is comprised of two domains separated by a nuclear localization sequence (NLS) (Figure 1.6; Kreppel et. al., 1997; Lubas et al., 1997). mRNA of OGT in mammalian cells are spliced leading to alternative transcripts. The more characterized nuclear cytoplasmic OGT (ncOGT), and the second, a mitochondrial OGT (mOGT). The N terminus of mOGT contains a mitochondrial localization sequence, which targets it to the mitochondria (Love et. al 2003).

The N terminus of OGT contains a protein-protein interaction domain known as a tetratricopeptide repeat (TPR) (Kreppel et al., 1997). The TPR domain of OGT is a 34 amino acid repeat that allows OGT to interact with itself and other proteins. Proteins that interact with OGT are known as OGT-interacting proteins (OIPs) (Iyer and Hart, 2003). OGT is modified by O-GlcNAc on its ser/thr residues and also by tyrosine phosphorylation (Kreppel et al., 1997). Through the TPR domain OGT can binding to itself and other OGT- interacting proteins. TPR motifs are found on proteins in a variety of organisms spanning from bacteria to humans (Blatch and Lassle, 1999). The number of TPR motifs in OGT varies from species to species. In C. elegans OGT has 13 TPR motifs while in rat OGT has 11.5 TPR motifs (Iyer and Hart, 2003).

The C terminal domain of OGT is the catalytic domain of this enzyme.
There has been debate in the literature determining the structure of OGT. OGT had first been purified by conventional and affinity chromatography from rat liver and shown to act as a trimer composed of two 110kDa subunits and one 78kDa subunit (Haltiwanger et al., 1992; Kreppel et. al, 1997; Lubas et al., 1997). Recent data has resolved the crystal structure of human OGT to be a homodimer (Jinek et al., 2004).

O-GlcNAc Transferase role in Arabadopsis development

In Arabidopsis OGT is encoded by two genes, spindly (spy) and secret agent (SEC). spy and sec are essential for regulating the Giberellin (GA) biosynthetic pathway (Jacobsen et al., 1996). GA is a plant hormone required at most stages of plant development that is essential for proper growth and development (Hedden and Phillips, 2000). Plants deficient in GA have severe developmental defects such as the dwarfism of the plants organs (Hedden and Phillips, 2000).

Depletion of spy leads to plants exhibiting a stretched, elongated phenotype. This resembles a plant that has been exposed to higher doses of GA, which also exhibits an elongated phenotype. In epistasis tests loss of spy function rescues the GA mutant phenotype, showing that spy negatively regulates the GA pathway (Jacobsen et al., 1996). Recent studies in Arabidopsis have shown that overexpressing full-length spy under the control of viral 35S promoter suppresses GA signaling in germinating seeds. Therefore, the full-length construct of spy can act as a dominant negative. Expressing the full-length construct also increases sensitivity to GA in the vegetative and reproductive tissues of the plant (Filardo and Swain, 2003). This suggests OGT can act differently in different tissues. When the spy TPR domain alone was overexpressed, plants were
slightly elongated displaying a weak phenocopy of a *spy* mutant (Filardo and Swain, 2003). Therefore the TPR mediates trimerization and is also sufficient to act as a dominant negative.

A double *sec;spy* double mutant lacks all OGT function and is embryonic lethal (Hartweck et al., 2002). This shows that it is possible to use a genetic approach to study the role of O-GlcNAc in a multicellular organism. Furthermore, O-GlcNac Transferase, even though it is a ubiquitous enzyme can modulate a specific signal transduction pathway.

While OGT has been studied extensively in plants, little is known about its role in animal development. When OGT is deleted in mouse embryonic stem cells (ES cells), the ES cells fail to divide resulting in their death (Shafi et al., 2000). This suggests that OGT plays an essential role in early development (Shafi et al., 2000). OGT is expressed in all areas of the mouse brain at all stages of development, as indicated by western blot and immunofluorence against OGT (Rex-Mathes et al., 2001). These results suggest that OGT is required for the function of cells in the nervous system and of OGT’s possible role in mouse brain development. Although this data suggests some role for OGT in animal development there is still not much known.

**OGT and E-cadherin**

While studying the role of cellular adhesions during the ER cell death pathway it was shown that both E-cadherin and β-catenin are O-Glycosylated early during apoptosis (Zhu et al., 2001). O-GlcNAc modified newly synthesized E-cadherin is not transported to the cell surface or assembled into the E-cadherin cytoskelton complex, leading to a loss of intercellular adhesion
It is thought that the addition of the O-GlcNAc modification may alter the conformation of E-cadherin allowing for it not to bind to p120-catenin anymore. E-cadherin molecules not modified by O-GlcNAc are still transported to the cell surface indicating a direct role for the modification in regulating cell surface transport of E-cadherin (Zhu et al., 2001). β-catenin however does not lose its adhesive functions when O-GlcNAc modified (Zhu et al., 2001). With E-cadherin as a target of OGT we can examine if OGT plays a role in zebrafish epiboly via the E-cadherin pathway.

**OGT modifies various cytoskeletal proteins**

OGT also modifies microtubule associated proteins (MAP). One such example is the MAP tau, which is responsible for organizing the microtubules on an axon in a neuron. Tau is found to be heavily glycosylated in neurofilaments purified from rat spinal cord (Dong et al., 1993). This along with evidence of OGT modifying other cytoskeletal associated proteins suggests a role for OGT coupling with microtubules during zebrafish epiboly.

**Zebrafish O-GlcNAc Transferase**

Like Arabidopsis, zebrafish has two copies of OGT. Both copies of OGT are found on chromosome 14 with a total of six transcriptional variants, four from copy I and two from copy II (Sohn and Do, 2005). Variants I and II contain a specific exon 19 generated by alternative splicing allowing for the insertion of a 16 amino acid sequence not found in mammalian OGT, or
the remaining 4 zOGT variants (Sohn and Do, 2005). The number of variants of OGT has enabled me to study the gene without depleting the fish completely of OGT leading to its death.

**O-GlcNAcase**

O-GlcNAcase is a β-N-acetylglucosaminidase responsible for catalyzing the removal of the O-GlcNAc modification (Figure 1.6; Dong and Hart, 1994; Kreppel et al. 1997). O-GlcNAcase acts as a monomer and is localized to the cytoplasm and nucleus (Wells et al. 2002). The cloned cDNA encodes a polypeptide of 916 amino acids with a predicted molecular weight of 103kDa (Gao et al. 2001).

Like OGT, O-GlcNAcase is also expressed in all tissues, and is predominantly expressed in the brain (Wells et al., 2002; Gao et al., 2001). It is also highly conserved from humans to C. elegans (Gao et al 2001). It has two functional domains consisting of an N-terminal hexosaminidase domain, and a C-terminal histone acetyltransferase (HAT) (Fig 1.6).

Both enzymes OGT and O-GlcNAcase are highly conserved from C. elegans to humans (Wells et al., 2001). The localization of both enzymes to the cytoplasm allows for rapid post-translational modification of their substrates (Vosseller et al., 2001).

**N-acetylglucosamine (O-GlcNAc)**

The role of common posttranslational modifications such as phosphorylation and proteolysis in regulating development through signal transduction has been extensively explored. Much is known about the proteins the common simple monosaccharide N-acetylglucosamine (O-
GlcNAc) modifies (Hart, 1997). However, little is known about its role in vivo in animal development. My work concentrates on establishing the role O-GlcNAc transferase (OGT) plays in the developing zebrafish embryogenesis.

O-GlcNAc modifies various nucleocytoplasmic proteins on their serine/threonine residues. O-GlcNAc modifies a wide variety of proteins modified ranging from nuclear proteins such as nuclear pore proteins, RNA polymerase II, various transcription factors, c-myc, and the p53 tumor suppressor to cytoskeletal proteins such as Tau, Talin, and Vinculin. (Torres and Hart, 1984; Hanover et al., 1987; Holt et al., 1987; Roquemore et al., 1996; Hart, 1997; Comer and Hart, 2000; Wells et al., 2001). O-GlcNAc sites have been mapped to residues that are targets of serine/threonine kinases or residues that are nearby serine/threonine phosphorylation sites. This suggests that O-GlcNAc and phosphorylation may have opposing effects on protein activity (Chou et al., 1995; Cole and Hart, 1999; Griffith and Schmitz, 1999; Cheng et al., 2000; Comer and Hart, 2000; Gao et al., 2001; Vosseller et al, 2001). Consistent with this, when Protein Kinase C (PKC) or Protein Kinase A (PKA) is activated total O-GlcNAc levels decrease in cultured cerebellar neurons. However, when PKC or PKA is inhibited total O-GlcNAc levels increase in cultured cerebellar neurons (Griffith and Schmitz, 1999). Therefore when phosphorylation levels decrease glycosylation levels increase in vivo. This raises the question does O-GlcNAcylation and phosphorylation act reciprocally.

In order for a post-translational modification (PTM) to be a regulator of signal transduction it has to be inducible and dynamic. O-GlcNAc fits these criteria. For example, O-GlcNAc displays inducible qualities since its removal or attachment to certain proteins can
change in response to stimuli such as insulin resistance (Wells et al., 2001). 2-acetamido-2-deoxy-D-glucopyranosylidene (PUGNAc) is a pharmacological inhibitor of O-GlcNAcase preventing the cycling of O-GlcNAc on proteins (Haltiwanger et al., 1998). Cells treated with PUGNAc increases O-GlcNAc levels and causes insulin resistance (Vosselar et al., 2002). There is increased O-GlcNAc modification on proteins such as β-catenin and insulin receptor substrate 1 (IRS1) in response to PUGNAc-induced insulin resistance, however it is not known if addition of O-GlcNAc alters the activity of the protein it modifies (Vosselar et al., 2002).

O-GlcNAc also demonstrates dynamic qualities that are necessary for the regulation of signal transduction. αβ-crystallin, a small heat shock protein is modified by O-GlcNAc at Thr 170 (Klemenz et al., 1991). Metabolic pulse-chase labeling assays showed that the modification degraded with a half life of 10 hours. This is compared to the modified protein which had a half life of 13.7 days (Roquemore et al., 1996). The turn over rate of the modification is faster than the turn over rate of the protein being modified, consistent with a role for O-GlcNAc regulating αβ-crystallin activity. This raises a question of comparing O-GlcNAc to phosphorylation turn over during signal transduction. Phosphorylation of αβ-crystallin may modify the function of the protein. It is also thought that addition of O-GlcNAc may also play a role in the heat shock response. The action of the enzymes involved in the removal and attachment of O-GlcNAc, O-GlcNAc case and O-GlcNAc Transferase (OGT) respectively (see Figure 1.7), may be analogous to the phosphatases and kinases involved in the phosphorylation of serine/threonine residues.

The addition of this modification to such a variety of proteins implies O-GlcNAc functions in many different processes. One target of O-GlcNAc is the carboxyl terminal domain
of RNA polymerase II (Kelly et al., 1993). O-GlcNAc modifies RNA polymerase II allowing it to exist in three states, phosphorylated, un-phosphorylated and also glycosylated. During elongation, RNA pol II is phosphorylated and has to be de-phosphorylated before it interacts with the promoter for the next round of transcription. However, in the pre-initiation complex RNA pol II is glycosylated recruiting initiation factors to the RNA polymerase complex (Kelly et al., 1993). This raises the possibility that O-GlcNAc may be involved in cyclic interactions of RNA polymerase II by controlling and regulating the transition to elongation. Another protein modified by O-GlcNAc is the oncogene c-myc, which regulates gene transcription in cell proliferation, cell differentiation and programmed cell death (Chou et al., 1995). c-myc is modified by O-GlcNAc at Threonine 58, which is a known phosphorylation site. Glycosylation and phosphorylation at this particular amino acid regulates the function of c-myc (Chou et al., 1995). This implies a role for O-GlcNAc in transcription.

O-GlcNAc also plays a role in translation. O-GlcNac modifies p67, a polypeptide that binds to and inhibits the phosphorylation of eukaryotic initiation factor 2 α (eIF2α) by eIF2 kinase (Datta et al., 1989; Chakraborty et al., 1994). When modified by O-GlcNAc p67 protects eIF2α from eIF2 kinase promoting protein synthesis (Datta et al., 1989; Chakraborty et al., 1994). O-GlcNAc also modifies Synapsin 1 which anchors synaptic vesicles to the cytoskeleton at pre-synaptic vesicles. The modification occurs on serine/threonine residues that are adjacent to phosphorylation sites. These sites can be as close as ten amino acids from known phosphorylation serine/threonine residues (Cole and Hart, 1999). Although, O-GlcNAc may not directly modulate the phosphorylation of synapsin 1, it may still regulate its function. O-GlcNAc is involved in a variety of different functions, only a few have been described here.
The role of O-GlcNAc Transferase in zebrafish Embryogenesis

I am using zebrafish as a model system since development is rapid and morphogenic processes are easily observed. Genetic tools such as knock down antisense morpholinos and mutant strains are also available to use. Zebrasfish can be easily manipulated with the ability to overexpress genes by injecting into embryos. O-GlcNAc Transferase controls ubiquitous reactions potentially affecting the activity of many proteins. Its role in animal development has been difficult to determine due to the inability to generate mouse knockout ES cells (Shafi et al., 2000). Using zebrafish embryos as a model to understand the role of OGT and its purpose in cell signaling will allow for a better understanding of the importance of glycosylation in early vertebrate development. Overexpression and loss of function assays show OGT is involved in patterning defects in embryos. I have identified and characterized two variants, variant II and variant IV of the zebrafish OGT ortholog and have subsequently examined the role they play in early vertebrate development.

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Figure 1.1. Schematic of a zebrafish embryo. In a zebrafish embryo the blastomeres site on top of the yolk cell. The yolk syncitial layer (YSL) is formed at about 3hpf when the blastomeres that are in direct contact with the yolk cell collapse and release their nuclei and cytoplasm into the adjacent yolk cell.
Figure 1.2. Map of the three presumptive germ layers of the zebrafish. *yellow*=yolk; *green*=endoderm and mesoderm; *blue*=mesoderm; *orange*=ectoderm *margin*=where the dividing cells meet the yolk.
Figure 1.3. The cytoskeleton of the zebrafish embryo. (A). There are two arrays of microtubules extending from the YSL in the zebrafish. The first is an internetworking within the YSL and the second extends from the YSL along the animal-vegetal axis of the yolk. (B). The presence of an F-actin within the YSL ring below the margin promotes contraction of the YSL during zebrafish epiboly. There is also an accumulation of actin at the vegetal end of the embryo.
Figure 1.4. The zebrafish embryo. (A). Fate map of the of the zebrafish embryo. *yellow* = yolk; *green* = endoderm and mesoderm; *blue* = mesoderm; *orange* = ectoderm. *margin* = where the dividing cells meet the yolk. (B). Live embryo at 6hpf. At about 6hpf the zebrafish organizer is formed at the dorsal side of the embryo (arrowhead). (C). Live embryo at 24hpf. The head, trunk and tail are shown.
Figure 1.5. Diagram of Nodal signaling pathway and BMP signaling pathway. Ligand binds to a receptor, activating it by a kinase. This phosphorylates transcription factors known as Smads. Smads get translocated to the nucleus to activate or repress downstream genes.
Figure 1.6. Structure of O-GlcNAc Transferase (OGT) and O-GlcNAcase (A). OGT is comprised of two domains separated by a nuclear localization sequence (NLS). The N terminal domain is comprised of a protein-protein domain known as a tetratricopeptide repeat (TPR). The C terminal domain is the catalytic unit of the enzyme. (B). O-GlcNAcase has two functional domains consisting of an N-terminal hexosaminidase domain, and a C-terminal histone acetyltransferase (HAT).
Figure 1.7. OGT and O-GlcNAcase are responsible for the addition and removal of the O-GlcNAc modification. O-GlcNAc binds to proteins on the SER/THR residues and may be analogous to the phosphotases and kinases involved in phosphorylation. O-GlcNAc may occupy similar, or adjacent SER/THR as in phosphorylation.
CHAPTER 2

O-LINKED GLYCOSYLATION REGULATES ENDODERM FORMATION AND MORPHOGENESIS IN ZEBRAFISH EMBRYOS

1Danielle M. Webster and Scott T. Dougan. To be submitted to BMC Developmental Biology
Abstract

Background

The post-translational addition of the simple monosaccharide O-linked b-N-acetylglucosamine (O-GlcNAc) is a dynamic and reversible modification found on serine and threonine residues in a wide variety of proteins. Unlike classical glycosylation, O-GlcNAc is a regulatory modification of cytoplasmic and nuclear proteins. Significant questions remain, however, about how addition of O-GlcNAc alters protein activity and affects animal development. A single enzyme, called O-GlcNAc Transferase (OGT), catalyzes the addition of O-GlcNAc to all known target proteins and complete loss of OGT function is lethal to mouse embryonic stem cells. In zebrafish, there are two OGT genes, encoding six different enzymatic isoforms that are expressed maternally and zygotically.

Results

We show that in zebrafish, OGT is ubiquitously expressed until the end of gastrulation, after which it becomes restricted to the brain. Overexpression of OGT results in embryos with shortened body axes and reduced brains at 24hpf. Loss of OGT function resulted in similar, but milder, defects. Extensive marker analysis in both gain- and loss-of-function embryos indicates that most derivatives of the mesoderm and ectoderm germ layers are present, but are often disorganized. By contrast, endoderm is severely reduced or absent. We did not observe any defects in the initial specification of the germ layers, suggesting that OGT activity is required for a later step in endoderm differentiation. OGT overexpression also disrupted cell adhesion in blastulae, delayed epiboly, and caused a severe disorganization of the microtubule and actin based cytoskeleton in the extra-embryonic yolk syncytial layer (YSL). The defects we report are similar in character to those reported for embryos completely lacking function of the Pouf1/Oct4 transcription factor spiel ohne grenzen.
Conclusions

We present here the first characterization of the function of OGT during vertebrate development. We conclude that O-GlcNAc regulates one or more proteins involved in endoderm differentiation and morphogenesis of mesoderm and ectodermal tissues. Potential targets include E-cadherin and the Pouf1/Oct4 transcription factor *spiel ohne grenzen*. Because overexpression of OGT caused defects similar to those obtained from OGT loss-of-function studies, we conclude that the cycling of O-GlcNAc on and off target proteins is essential for developmental processes.

Background

It is well documented that post-translational modifications such as phosphorylation, proteolytic cleavage and glycosylation, control key steps of embryogenesis by modulating the activities of developmentally relevant proteins. For example, N-linked glycosylation affects the activities of the Notch receptor and the secreted Wnt ligands [1, 2]. This type of glycosylation is irreversible and occurs in the lumen of the rough endoplasmic reticulum and golgi apparatus. By contrast, the O-linked addition of the simple monosaccharide β-N-acetylglucosamine (O-GlcNAcylation) occurs on nuclear and cytoplasmic proteins and is reversible [3]. Attachment of O-GlcNAc is induced in response to external stimuli such as nutrient status and stress [4]. These characteristics suggest that O-GlcNAc is a regulatory post-translational modification (PTM) [5]. Many of the residues modified by O-GlcNAc are also targets of ser/thr kinases, suggesting that this form of glycosylation acts antagonistically to phosphorylation [6]. Over 500 nuclear and cytoplasmic proteins are modified by O-GlcNAc, including NeuroD1, β-catenin, c-myc and the cytoplasmic tails of plakoglobin and E-cadherin [7-11]. This list of targets suggests that O-GlcNAc may control important developmental processes, but the *in vivo* role of O-GlcNAc remains elusive.
Only two enzymes, O-GlcNAc transferase (OGT) and O-GlcNAcase, respectively, catalyze the addition and removal of O-GlcNAc to all known targets [12, 13]. O-GlcNAcase is a bifunctional enzyme that also has histone acetyl transferase activity [13]. OGT peptides consist of a C-terminal catalytic domain and an N-terminal tetratricopeptide repeat (TPR) domain, which mediates protein-protein interactions [14]. Although the monomer can catalyze the addition of O-GlcNAc in *in vitro* assays, OGT trimers have a greater affinity for the UDP-GlcNAc substrate [14]. This suggests that OGT forms an active multimeric complex *in vivo*. In support of this idea, the TPR domain is essential for enzyme activity in *Xenopus* oocytes [15].

Functional studies have focused mainly on how O-GlcNAc modification alters protein activity *in vitro* or in cell culture. These studies have implicated O-GlcNAc in the regulation of transcription, protein stability and cell cycle progression, among other events [16-19]. Analysis of *ogt* function in whole animals, however, has been hampered by its requirement for cell division. In mouse embryos, complete loss of *ogt* function prevents division of embryonic stem cells, fibroblasts, T-cells and male gametes [20, 21]. In *Xenopus* oocytes, the use of blocking antibodies demonstrated that OGT activity is required for oocyte maturation and entry into M-phase [15, 22]. Finally, there are two *ogt* genes in *Arabidopsis*, called *spindly* (*spy*) and *secret agent* (*sec*) [23]. *spy; sec* double mutant seeds fail to germinate, indicating that the two genes have overlapping requirements in early development [24]. Thus, *ogt* function is absolutely required for cell proliferation in mice, frogs and plants. By contrast, *ogt* is not required for cell proliferation in *C. elegans* and *ogt* mutant worms have aberrant insulin-like signalling [25]. Similarly, *spy* single mutants survive, and the resulting plants display an aberrant response to the plant growth hormone, gibberellin [26, 27]. This demonstrates that it is possible to identify roles of *ogt* in specific signalling pathways when the requirement for cell proliferation is circumvented.
Zebrafish is an ideal model organism in which to study the role of OGT during embryonic development. There are two *ogt* orthologues in zebrafish, present in tandem copies on linkage group 14, and both genes are expressed maternally [28]. There are two alternatively spliced exons in zebrafish *ogt*, which results in the production of six different enzyme isoforms. We have adopted two approaches to understand the function of OGT during embryogenesis. We show that full-length and short isoforms of OGT are both active *in vitro* and *in vivo*, in contrast to previous results [28]. Our data indicates that both addition and removal of O-GlcNAc is essential for endoderm differentiation, rather than its initial specification, and for the normal organization of mesoderm and ectodermal tissues. These defects are reminiscent of those described for embryos lacking the maternal and zygotic function of *spiel ohne grenzen* (*spg*)/Oct4. In addition, overexpression of OGT disrupted cell adhesion and delayed progression through epiboly. We conclude that OGT plays a critical role during zebrafish embryogenesis, but further work is necessary to determine which are the essential targets modified by O-GlcNAc. This work emphasizes the importance of considering the potential role of O-GlcNAc in regulating the activity of developmentally important proteins, in addition to other reversible PTMs like phosphorylation.

**Results**

*Two ogt genes arose from a zebrafish specific gene duplication*

Zebrafish has two *ogt* genes, which produce six different transcripts via alternate splicing (Fig. 1A) [28]. The splice variants contain significant sequence overlap and the two zebrafish genes are 67% identical at the nucleic acid level, with 89% amino acid identity. *Arabidopsis thaliana* is the only other genetic organism with two *ogt* genes, called *spindly* (*spy*) and *secret agent* (*sec*) [23]. This raises the question of whether the zebrafish and *Arabidopsis* genes originated from the same duplication event, or if there were independent
duplication events in each species. A sequence alignment and phylogenetic analysis showed that *Danio rerio* ogt copy I is 37% identical to sec at the amino acid level and only 21% identical to spy (Fig. 1B). Similarly, *Danio rerio* ogt copy II has 39% identity with sec, and 22% identity with spy (Fig. 1B). Since both zebrafish genes are more closely related to sec than spy, this indicates that the genes arose from independent duplication events in each species. Worms, sea squirts, mice and humans each have only one ogt gene (Fig. 1B). This raises the possibility that the ogt gene duplication occurred during a teleost specific whole genome duplication event specific [29]. To test this idea, we examined the sequenced genomes of other teleost fish for ogt orthologues. The green spotted pufferfish (*Tetraodon nigroviridis*), fugu pufferfish (*Takifugu rubripes*), stickleback (*Gasterosteaus aculeatus*) and medaka (*Oryzias latipes*) each have only a single ogt orthologue (Fig. 1B). We conclude that the *D. rerio* copy I and copy II arose from a recent zebrafish specific gene duplication.

To investigate the function of OGT in zebrafish, we isolated a full-length ogt transcript from a 15-19 hours post fertilization (hpf) cDNA library. Sequence analysis revealed that this transcript encodes the variant 2 OGT isoform, which lacks exon 2a and contains exon19 (Fig. 1A) [28]. Since bacterially expressed variant 2 OGT lacked enzymatic activity in *in vitro* assays, we removed exon 19 in order to generate an active enzyme with the same sequence as the variant 4 isoform (Fig. 1A) [28]. We expressed our constructs in CHO-IR cells in order to determine if they encoded enzymatic active proteins. In cells expressing the synthetic variant 4 isoform, O-GlcNAc levels are increased significantly as compared to mock transfected control cells, but are not as high as in cells expressing a human OGT construct (Fig. 1C, lane 1-3). Surprisingly, cells expressing variant 2 OGT also have increased O-GlcNAc (Fig. 1C, lane 4). Thus, in contrast to previous reports, both OGT isoforms have enzymatic activity [28]. Attempts to determine the activity of the enzymes in whole embryos failed because the existing O-GlcNAc specific antibodies did not work on
zebrafish cell extracts.

**ogt expression is gradually restricted to the brain**

Previous results indicated that *ogt* is expressed continuously from oogenesis through 5 days post-fertilization (dpf) [28]. We extended these studies by examining the spatial distribution of *ogt* transcripts by *in situ* hybridization in a developmental time-course (Fig. 1D-L). Our probe was complementary to the entire variant 2 sequence and therefore is not expected to differentiate between the six different *ogt* transcripts. *ogt* is expressed ubiquitously from the cleavage stages through the end of gastrulation (Fig. 1D-G). This staining was specific, since no signal was detected with a sense probe (Fig. 1L). Beginning at 24 hpf, *ogt* transcripts become restricted to neural tissues in the head and trunk (Fig. 1H). Over the next two days of development, transcripts become progressively restricted to the brain (Fig. 1I-K). This expression pattern is consistent with an exclusive role for OGT in neural development or function after gastrulation, and a more general role in all cells at earlier stages.

**ogt function is required for proliferation and morphogenesis of ectodermal, mesodermal and endodermal tissues**

To determine the function of *ogt*, we designed two non-overlapping, translation-blocking morpholino oligonucleotides (MOs) that are complementary to sequences present in all six alternatively spliced transcripts. To characterize the effectiveness of the MOs, we fused the 5’ 1500 bp of the variant 4 cDNA to the GFP coding region. After co-injecting OGT-GFP mRNA with a control MO into embryos at the 1-4 cell stage, the fluorescent fusion protein is ubiquitously expressed in all embryos (Fig. 1M; N=30). In the presence of MO1, fluorescence levels are not detectable above the background autofluorescence of the yolk (Fig. 1N; N=30). This indicates that MO1 can effectively block translation of *ogt*
transcripts. Similar results were obtained with MO2 (data not shown).

Next, we examined the consequences of depleting *ogt* function from embryos. At 24
hpf, embryos injected with 7.5pg of a control MO developed normally, and the eyes, midbrain-hindbrain boundary, notochord and somites are clearly visible (Fig. 2A; N=40). By contrast, a more than a third (34%) of embryos injected with 7.5pg MO1 or MO2 (37%) had shortened body axes, reduced heads and twisted notochords (Fig. 2B, C; N=32/94, N=35/95, respectively). Some embryos showed signs of cell death in the head, which became more pronounced and more frequent with age (data not shown). We next examined the possibility that this altered morphology was caused by defects in cell fate specification or differentiation by examining the expression of genes that mark derivative of the neurectoderm, mesoderm or endoderm. *emx1* marks the dorsal telencephalon in embryos injected with the control MO (Fig. 2D) [30]. The dorsal telencephalon is present in embryos injected with MO1 (N=20) or MO2 (N=25), but is reduced, as indicated by the smaller *emx1* expression domain (Fig. 2E, F). The epiphysis is a structure of the dorsal diencephalon that expresses the *floating head* (*flh*) transcription factor (Fig. 2G) [31]. *flh* is expressed normally in the forebrains of *ogt* morphants (MO1, N=30; MO2, N=30), indicating that the epiphysis is present (Fig. 2H, I). In the segmented hindbrain, rhombomeres 3 and 5 express the zinc finger transcription factor *krox20* (Fig. 2J) [32]. As in controls, *krox20* is expressed in two stripes in the morphants, although expression in rhombomere 3 is often reduced (MO1, N=30; MO2, N=27) (Fig. 2K, L). We conclude that anterior-posterior patterning of the brain is normal, but the amount of tissue is reduced in the forebrain and hindbrain.

Derivatives of the mesoderm and endoderm are also reduced when *ogt* function is depleted. Within the mesoderm, slow muscle forms in the somites on either side of the midline, and expresses *α-tropomyosin* (Fig. 2 M) [33]. In the morphants, *α-tropomyosin* expression reveals the presence of slow muscle tissue in a reduced number of highly
disorganized somites, as compared to embryos injected with the control MO (Fig. 2N, O; MO1, 19/30; MO2, 15/25). *axial/foxA2* is expressed in the presumptive notochord as well as in the endoderm precursors (Fig. 2R)[34]. Expression of *axial/foxA2* is greatly reduced at the midline, indicating a reduction in notochord (Fig. 2S). Endoderm precursors are apparent during gastrulation, as individual *sox17* and *axial/foxA2* expressing cells migrating toward the animal pole (Fig. 2P, R) [35]. In addition, *sox17* is expressed in a small group of dorsal forerunner cells (Fig. 2P, arrow). Endodermal expression of both genes is significantly reduced in the morphants, indicating that they have significantly fewer endoderm precursors than embryos injected with control MOs (Fig. 2Q, 22/30; S, 20/30). The morphants also have fewer dorsal forerunner cells (Fig. 2Q). We conclude that *ogt* is required for the normal organization of the germ layers. In addition, the reduction of ectodermal and endodermal tissues is consistent with a role for *ogt* in cell proliferation.

Alternately, the reduction in tissues could be explained by a defect in cell fate specification. To test this, we examined pregastrula stage embryos for the expression of early mesoderm and endoderm markers. In 5 hpf controls, the *brachyury* homologue *no-tail* (*ntl*) is expressed in a marginal ring that includes all mesoderm and endoderm precursors, as in wild type (Fig. 3E) [36]. This expression is not disrupted in by injection of MO1 (Fig. 3F, N=25) or MO2 (data not shown). Within the dorsal mesoderm, *flh* is expressed in the presumptive notochord (Fig. 3A), while *goosecoid* is expressed in the prechordal plate (Fig. 3C) [36, 37]. Both genes are expressed normally in the morphants (Fig. 3B, N=25; 3D, N=25). Finally, the *mezzo* transcription factor acts downstream of Nodal signals to specify endoderm, and is expressed in a ring at the margin (Fig. 3G) [38]. *mezzo* expression is normal in the morphants (Fig. 3H, N=30). Since the initial specification of mesoderm and endoderm appears normal when *ogt* levels are decreased, we conclude that the reduction of derivatives of the endoderm, mesoderm and ectoderm at later stages in *ogt* morphants is caused by the
failure of precursor cells to proliferate. The shortened body axes and disorganized tissues apparent in 24 hpf embryos could indicate a role for OGT in controlling morphogenetic cell movements.

**OGT overexpression disrupts ectoderm, mesoderm and endoderm tissues.**

Both the addition and removal of O-GlcNAc to target proteins is essential in other contexts, such as the control of glucose metabolism [5]. To determine the effects of increasing O-GlcNAc levels on embryos, we injected 500pg mRNA encoding variant 4 or variant 2 OGT mRNA at the 1-4 cell stage. Embryos injected with 500pg of β-galactosidase mRNA were indistinguishable from wild type, indicating that the introduction of such a large quantity of mRNA did not cause non-specific defects (Figure 4A, B). By contrast, injection of mRNA encoding variant 4 OGT generated three classes of embryos at 24 hpf. Class I embryos displayed mildly reduced heads, eye and notochords, resulting in a shortened body axis similar to that observed in embryos injected with OGT MOs (Figure 3E; 23/129; compare with Fig. 2B, C). Class II had more severe defects, including greatly reduced heads and no distinguishable body axis (Figure 4F; 20/129). Finally, class III embryos were indistinguishable from control embryos injected with β-galactosidase mRNA (data not shown; 35/129). The remaining 51 embryos died soon after gastrulation (see below). Lower doses of OGT mRNA produced no defects. Embryos injected with mRNA encoding OGT variant 2 enzyme exhibited similar defects at similar frequencies (Figure 4C, D), consistent with our results showing this variant is active in CHO cells (Fig. 1C).

We performed marker gene analysis on OGT overexpressing embryos to determine which cell types were present at 24 hpf. The dorsal telencephalon is mildly expanded in Class I embryos (Fig. 5H, N=20) and is expanded to a greater extent in Class II embryos (Fig. 5O), as compared with β-galactosidase mRNA injected embryos (Fig. 5A). The epiphysis in Class I embryo is comparable in size to that observed in embryos injected with β-
galactosidase mRNA, as revealed by flh expression (Fig. 5B, I; N=20). Class II embryos, by contrast, have an extremely small epiphysis, as indicated by the strong reduction in flh expression (Fig. 5P). The transcription factor pax2.1 is expressed at the mid-brain, hindbrain (MBHB) boundary as well as in the otic vesicles (Fig. 5D) [39]. The otic vesicles and MBHB boundary are present and express pax2.1 in class I embryos, although the ears are abnormally positioned (Fig. 5K; 10/20). By contrast, the MBHB boundary is disrupted in Class II embryos, and the otic vesicles are reduced, as indicated by pax2.1 (Fig. 5R; 5/20). In the hindbrain, the stripes of krox20 are smaller in Class I (Fig. 5J; N=25) and Class II (Fig. 5Q) embryos. Thus, rhombomeres 3 and 5 are reduced in comparison to β-galactosidase expressing embryos (Fig. 5C). This data demonstrates that overexpression of OGT does not disrupt patterning of the neural tube along the anterior-posterior axis in OGT overexpressing embryos, but does reduce the amount of tissues in the forebrain, mid-brain and hindbrain. This is similar to the defects we observed in OGT morphants. Furthermore, OGT expressing Class I and II embryos have fewer endoderm precursors than β-galactosidase expressing embryos, as revealed by expression of axial/foxA2 (Fig. 5E, L, S; N=15/20) and sox17 (Fig. 5F, M, T; N=16/20). The dorsal forerunners are greatly reduced or absent (Fig. 5F, M, T).

Derivatives of the mesoderm are also reduced in OGT overexpressing embryos. The hatching gland is a derivative of the prechordal plate and can be detected at 12 hpf by expression of the marker hgg1 (Fig. 5G)[40]. The hatching gland is greatly reduced in Class I (Fig. 5N; 12/20) and Class II (Fig. 5U) embryos. This decrease in hgg1 expression is the result of fewer cells in the hatching gland, as indicated by examining hgg1 expression at 18 hpf, when individual cells can be distinguished. Within the trunk, a-tropomyosin expression reveals a decreased number of disorganized somites in 24 hpf Class I (Fig. 6H, 14/20) and Class II (Fig. 6I) embryos, when compared to β-galactosidase expressing embryos (Fig. 6G). Examination of MyoD expression, an early somite marker (Fig. 6D), reveals that the defects
in somite number and organization can be detected at the earliest stages of somite formation (Fig. 6E, F; N=15/20)[41]. The notochord is similarly reduced and disorganized at 8 hpf, as revealed by the midline expression of axial/foxA2 (Fig. 5L, S). Defects in notochord formation are more apparent at the end of gastrulation. In OGT expressing embryos, ntl expression does not extend along the entire body axis (Fig. 6A-C; N=10/20). This could indicate a defect in morphogenetic movements in addition to the decreased number of cells.

**OGT overexpression causes delays in epiboly and decreases cell adhesion**

A significant number of OGT overexpressing cells die before 24 hpf, and many embryos examined before this stage had signs of defective morphogenesis. To examine this more carefully, we examined the development of living embryos over a time course from 3-12 hpf. OGT overexpressing embryos develop at the same rate as β-galactosidase expressing embryos until 3 hpf (Fig. 7A, E). Occasional blastomeres separate from the blastoderm of OGT overexpressing embryos during this period, indicating defects in cell adhesion (Fig. 8F; data not shown). Epiboly initiates at the same time in OGT overexpressing and β-galactosidase expressing embryos and they reach 40% epiboly by 5 hpf (Fig. 7B, F). In OGT overexpressing embryos, epiboly slows after gastrulation. At 8 hpf, β-galactosidase expressing embryos are at 80% epiboly while OGT overexpressing are still at 40% epiboly (Fig. 7C, F). OGT overexpressing embryos also had an accumulation of cells at the animal pole, suggesting a defect with morphogenesis or cell adhesion. (Figure 7F, arrow). At 12 hpf, when β-galactosidase expressing embryos are at the 3 somite stage, 40% of OGT overexpressing embryos begin to die (Fig. 7D, G; N=52/129). It is unlikely that defects in cell proliferation could explain a lack of cell adhesion or delayed epiboly. We conclude that OGT controls the activity of one or more proteins involved these morphogenetic processes.
OGT overexpression disrupts the cytoskeleton

We examined the cytoskeleton in order to understand the molecular basis for the epiboly and adhesion defects in OGT expressing embryos. First, we examined the microtubules in the yolk and yolk syncytial layer because they are required for epiboly [42]. At 4 hpf, a monoclonal antibody against acetylated tubulin reacts to a group of yolk microtubules that extends around the cortex deep into the yolk cell, and another, shorter organized latticework of microtubules within the yolk syncytial layer (YSL) (Fig. 8A) [42]. In OGT expressing embryos, by contrast, the microtubule lattice in the YSL is disrupted (Fig. 8B). In addition, the yolk microtubules are shortened and do not extend into the yolk (Fig. 8B). These microtubule defects could explain the delay in epiboly in OGT overexpressing embryos.

To examine the actin network, we stained β-galacotsidase and OGT expressing embryos with rhodamine-phalloidin. F-actin associates with the cytoplasmic domain of E-cadherin in the cortical cytoplasm, and decorates the cell membranes [43]. In mid-gastrula staged control embryos, the cells are roughly the same size and evenly spaced (Fig. 8C, E). In the YSL, a contractile band of F-actin is visible in rhodamine-phalloidin stained embryos (Fig. 8C, E) [44]. In OGT expressing embryos, cells are irregularly shaped and F-actin levels are decreased (Fig. 8D, 9/15, compare with 8C). In more severely affected (Class II) embryos, some cells detach from the blastoderm and reside in the yolk (Fig. 8F). In addition, the contractile band in the YSL appears expanded in both mild (Fig. 8D) and severely affected embryos (Fig. 8F, 10/15). The disruption of F-actin in the YSL could contribute to the delay in epiboly. Furthermore, the reduced F-actin in the blastomeres suggests that these embryos have decreased levels of E-cadherin, consistent with their lower adhesivity.
Discussion

Regulation of O-GlcNAc Transferase during zebrafish embryogenesis

We have presented the most detailed functional analysis of OGT function during development in any vertebrate. We identified and characterized the function of the enzyme OGT in zebrafish. We cloned one 1053a.a. variant, variant 2 out of a 15-19hpf cDNA library. Since this isoform was shown to by Sohn and Do to have no O-GlcNAc Transferase activity in in vitro assays, we deleted the unique exon of about 16a.a., exon 19, which was predicted to be inhibitory [28]. Surprisingly, we found that both isoforms of the enzyme are active in CHO-IR cells and in zebrafish embryos. The difference between our results could be explained by the fact we expressed our enzyme in eukaryotic cells whereas Sohn and Do used bacterially expressed protein. In addition, the cells we used may have OGT interacting proteins that facilitate enzymatic activity that are missing in the system used by Sohn and Do. OGT is expressed ubiquitously until about 8hpf, when expression begins to decrease. Expression is upregulated at about 24hpf, when it is restricted primarily to neural tissue. Thereafter OGT transcripts are progressively restricted to the anterior brain.

Manipulating levels of O-GlcNAc does not affect mesoderm and ectoderm induction

Embryos with reduced ogt function displayed a significant reduction in the derivatives of endoderm, mesoderm and ectoderm. We could detect no defects in germ layer specification, indicating that the tissue defects occurred later in development, probably due to reduced proliferation. Analysis of ogt function in the mouse was hampered by a strict requirement for ogt for the division of embryonic stem cells and other cell types. In our experiments, however, we did not completely eliminate ogt function. In zebrafish ogt is expressed maternally, including during oogenesis. MOs would not block translation of transcripts in oocytes or in the first stages of embryonic development.
O-linked glycosylation regulates endoderm formation

Zebrafish fate mapping shows endodermal progenitors are derived from cells closest to the margin [45]. The commitment of endodermal cells begins just after the onset of gastrulation [46,47]. Several signalling pathways such as the Nodal, FGF and BMP signalling pathways have been shown to be crucial players in the formation endoderm. Embryos lacking both maternal and zygotic function of speil-ohen-grezen (spg) have defects in endoderm formation [48,49]. Zygotic spg mutants have defects in the MBHB boundary. Similarly, embryos depleted of OGT show a marked reduction in endoderm precursors, as shown by decreased axial and sox17 expression. MZspg mutants also have cytoskeletal defects and delays in epiboly similar to OGT overexpressing embryos. This raises the possibility that OGT catalyzes the addition of O-GlcNAc to Spg/Oct4, and that O-GlcNAcylation controls the activity of Spg/Oct4.

O-GlcNAc Transferase plays a role in zebrafish epiboly

Previous studies have shown that cells accumulate on the dorsal side establishing and lengthening the embryonic axis during convergence and extension (Warga et al., 1990). In this study we show that the notochord are specified, however it does not fully extend along the dorsal axis of the embryo. OGT may regulate some protein(s) involved in convergence and extension.

When overexpressing zOGT we noticed a delay during epiboly. Embryos overexpressing OGT were slowed during epiboly and exhibited a phenotype of cells gathering on top of the yolk as a compacted ball. This ball of cells would also have a few cells falling off during gastrulation. This suggests a problem with cell-adhesion. Previous studies show that OGT modifies the cytoplasmic tail of E-cadherin (Zhu et al., 2001). O-GlcNAc modified E-
cadherin is not transported to the membrane (Zhu et al., 2001). This could result in the loss of cell adhesion in embryos overexpressing OGT.

Along with E-cadherin there are other factors that drive epiboly such as yolk microtubules and actin filaments. When overexpressing OGT and looking at acetylated tubulin we noticed that there was disorganization within the lattice work of yolk microtubules within the YSL and along the animal-vegetal axis of the embryo. Disrupted yolk microtubules during gastrulation leads to a delay in epiboly (Solnica-Krezel and Driever, 1994). OGT may modify microtubules or microtubule associated proteins resulting in disruption of the microtubules.

F-actin clearly marks the membranes of individual blastomeres. We show that there is a change in the shape of cells when zOGT is overexpressed. Embryos resemble the MZspg mutant which show rounded and disorganized cells during epiboly (Figure D; Lanchit et al., 2008). OGT may modify spg in embryos making it inactive.

**Conclusions**

We have cloned and characterized the zebrafish homolog of O-GlcNAc Transferase. We show that is is ubiquitously expressed up until 48hpf where begins to be restricted to the brain. Our data suggests a very broad role for zOGT during zebrafish embryogenesis. We have looked more closely at a few of these roles and we conclude that manipulating levels of zOGT lead to a reduction in endoderm. Lastly, overexpressing zOGT caused a delay in epiboly due to the disorganization of the cytoskeleton. Embryos overexpressing OGT are remarkably similar to MZspg mutants. Not all phenotypes exhibited by embryos with manipulated O-GlcNAc levels are similar to MZspg mutants. This is understandable as OGT
is a ubiquitous enzyme and modifies many proteins. *spg* is just one target of OGT that we have identified. We can use this to examine more closely the genetic and biochemical interaction between OGT and *spg*. These findings demonstrate that OGT can be linked to a specific pathway during animal development. The zebrafish is shown here that is can be used as a tool to examine the role of OGT in animals and provide a basis for examination of the role of O-glycosylation during animal development.

**Materials and Methods**

**Zebrafish strains and staging**

We used the WIK strain to obtain wild type embryos. In all experiments, the embryonic stages were determined by morphology and are reported as hours post fertilization (hpf) at 28.5°C, according to Kimmel et al., 1995.

**Identification of endogenous zebrafish OGT**

We cloned a transcript encoding the variant 2 isoform from a 15-19hpf cDNA library acquired from Bruce Appel at Vanderbilt University. I used primers obtained from the cloned sequence of the full length zOGT to identify and isolate the clone (Sohn and Do, 2005). The isolated clone was sequenced to ensure if was the correct gene of interest. Variant 2 was TOPO cloned into the XbaI site of the pCS2 plasmid. To generate variant 4, I deleted exon 19 from variant 2 by long extension polymerase chain reaction (PCR) using primers flanking exon 19. zOGT full-length F: 5’-TTACGTCGACGAATGGCGAGCT- CGGTG-3’, zOGT full-length R: 5’-ATGCGCGGCCGATCAGGTGCTCTCGC-3’, zOGTextF: 5’-TTTTTCTTAAGAAAAAGGCTGTTATTGACT-3’, zOGTextR: 5’-AAT- TAGCTGGGTACCAGGCCATGACATTGCC-3’, exon19F: 5’-GCCACCACACAGATTAAATAAA-3’, exon19R: 5’-TTTTTCCCCCGCGGCACATTTAAAAACCTCCC-3’.
Whole mount *in situ* hybridization


**Microinjection of mRNA**

Sense mRNA was made using the mMESSAGE mMACHINE RNA synthesis kit (Ambion). We injected 500 pg of *zOGT* or *β-galactosidase* mRNA into chorionated embryos at the 1-4 cell stage. Embryos were allowed to develop and examined at 24hpf or fixed at appropriate stages for *in situ* hybridization.

**Microinjection of antisense morpholino oligonucleotides (MO)**

We designed a translational blocking morpholino *zOGT* MO1. To rule out the possibility that the phenotype was not nonspecific, we designed 5 base pair mismatch (mis) MO for *zOGT* MO1. We also designed another morpholino, *zOGT* MO2, against the translational start site, not overlapping *zOGT* MO1. All MO were dissolved in distilled water at a concentration of 30mg/ml and stored at -20°C. The working stock of each MO was diluted to 7.5ng in 0.2M KCL prior to injection. Phenol red was added to the solution in order to trace the injection. Embryos were injected with 7.5ng *zOGT* MO1 at the 1-4 cell stage. As a control, both the mismatch MO and *zOGT* MO2 were injected at the same concentration. Embryos were allowed to develop and examined at 24hpf or fixed at appropriate stages for *in situ* hybridization. Two different combination of antisense morpholinos (MOs) were obtained from Gene Tool, Inc., Philomath, OR) for *zOGT* and their mismatch designed as followed.
Lower case letters indicates change in zOGT MOs sequence. zOGT MO1: 5’-
CCACGTTCCCA[CGAGCTG]CCAT-3’, zOGT MO2: 5’-
TCTCCTTCATCTTTACTGGATCTCT-3’, zOGT control: 5’-TCTTAGGTCATTTC-
TCACTTCTCTCT-3’. OGT-GFP was generated by PCR of a the first 1500bp of zOGT. This
was inserted into pGFPCS2. Primers for generation of OGT-GFP are as follows: OGT-GFPf:
5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCGAGCTCGGTGGGG-3’,
OGT-GFPf: 5’-GGGGACCACTTTGTACAAAAAAGCAGGCTTAATGGCGAGCTCGGTGGGG-3’,
ATGCAC-3’. 1500bpOGT was cloned into pGFPCS2 using gateway technology.

Labelling F-acting in fixed embryos

At 8hpf embryos were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS)
overnight at 4°C. Embryos were dechorionated and washed thoroughly with PBS containing
0.1% Tween (PBT). They were incubated in blocking solution containing 2% bovine serum albumin (BSA) at room temperature for 2 hours. To label F-Actin, embryos were incubated
with rhodamine-phalloidin at a dilution of 1:40 at room temperature in the dark. Embryos
were then washed thoroughly in PBT. Rhodamine-phalloidin was dissolved in methanol at a
concentration of 6.6µM and stored at -20°C.

Labelling microtubules in fixed embryos

Embryos were dechorinated and fixed at 4hpf with 0.5% Triton X-100 (Gard Fixative) for 4
hours at room temperature. They were washed once with PBS and stored overnight in 100%
methanol at -20°C. The following day embryos were washed with PBS three times and then
incubated in 3.7mg/ml NaBh₄ diluted in water for 6 hours at room temperature. They were
then washed 8 times in PBS and blocked for 1.5 hrs in blocking solution (1% DMSO, 0.5%
Triton, 2% BSA, 2% sheep serum). Embryos were incubated in primary antibody
(monoclonal 12G10 acquired from the Jacek Gaertig, UGA) at 1:100 dilution in block
solution overnight. The following day embryos were washed 3 times in PBS and incubated overnight in anti-mouse IgG-FITC (Zymed Laboratories) at a dilution of 1:200. Embryos were then washed with PBS and mounted.

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Figure 2.1. (A) Exons of v.2 and v.4 of zOGT. Amino acid structure of v.2 and v.4 zOGT. MO1 and MO2 designed against the UTR of zOGT. Displays of MO1 and MO2. (B) Phylogentic tree of OGT. (C) CHO-IR mammalian cells overexpressing zOGT and hOGT. (D-K) Expression pattern of zebrafish OGT from 1.25hpf to 72hpf using antisense probe against zOGT. (L) Control embryo at 1.25hpf with sense probe of zOGT. (M) 24hpf embryo injected with zOGT-GFP and control MO. (N) 24hpf embryo injected with zOGT-GFP and antisense MO against zOGT showing knock down of zOGT-GFP expression.
Figure 2.2. Depleting OGT causes morphological defects and a reduction in endodermal precursor cells. Embryos injected at the 1-4 cell stage with 500pg mRNA. (A-O) Embryos shown at 24hpf. (A) β-gal injected live; (B) Class I zOGT injected live; (C) Class II zOGT injected live. (D-L) Embryos were fixed at 24hpf and processed for in situ (D-F) emx1, (G-I) flh, (J-L) krox20, (M-O) α-tropomyosin. (P-T) Embryos fixed at 8hpf and processed for in situ. Embryos depleted of zOGT show a reduction in endodermal precursor cells.
Figure 2.3. Manipulating levels of O-GlcNAc does not affect early specification. Embryos were injected at the 1-4 cell stage and fixed at 5hpf. They were examined for the molecular markers $flh$ (A,B), $gsc$ (C,D), $ntl$ (E,F) and $mezzo$ (G,H). Mesodermal and endodermal progenitors were not affected by depleting levels of OGT.
Figure 2.4. Overexpressing zOGT causes similar defects as depleting levels of zOGT.

Embryos were injected at the 1-4 cell stage and examined at 24hpf. (A-B) β-gal injected. (C-D) Embryos were injected with v.2 zOGT. (E-F) Embryos were injected with v.4 zOGT.

Survivors were classified into two groups. Class I embryos exhibited a mild phenotype (C,E), while Class II embryos exhibited a more severe phenotype (D,F).
bgal

v.2 zOGT

v.4 zOGT
Figure 2.5. Overexpressing zOGT does not affect brain specification but causes a reduction in endoderm. Embryos were injected at the 1-4 cell stage with v.4zOGT and fixed and processed for *in situ*. We show that ectodermal cell types are still present after overexpressing zOGT as shown by the molecular markers *emx1* (A,H,O), *flh* (B,I,P), *krox 20* (C,J,Q) and *pax2.1* (D,K,R). Overexpressing zOGT also causes a reduction in precursor endodermal cells (L,M,S,T). Prechordal plate derivatives are specified, however its expression is altered (N,U).
Figure 2.6. Overexpressing zOGT results in defects during gastrulation. Embryos were injected at the 1.4 cell stage, fixed and processed for in situ. The notochord does not extend completely along the dorsal axis as shown by ntl expression (B,C). Somites are stretched and disorganized (E,F,H,I). pax2.1 expression was stretched and extended along the dorsal axis reflecting the altered body shape (K,L).
Figure 2.7. Overexpressing zOGT causes a delay in epiboly. Embryos injected with zOGT were examined over a time course until from 3hpf to 12hpf. At about 5hpf embryos exhibit an aggregation of cells on top of the yolk and migrate slower than control embryos (F,G,arrowhead). Embryos with this phenotype die by 12hpf (H).
Figure 2.8. Overexpressing zOGT disrupts the cytoskeleton. Embryos were injected at the 1-4 cell stage. Embryos were fixed and acetylated tubulin was antibody stained with 12G10 antibody (A,B). Yolk microtubules were disorganized along the animal vegetal axis (B). Embryos were also stained with rhodamine-phallodin (C-F). Embryos exhibited a decrease in cell-cell adhesion as shown by changes in cell shape (D) and blastomeres falling away from the yolk (F).
CHAPTER 3

CONCLUSION

This data supports the idea that it is possible to use a genetic approach to study the role of OGT in a multicellular organism. Since there are two OGT genes in zebrafish, it is possible to study its role without completely depleting the organism of OGT. This allows for manipulation of O-GlcNAc without resulting in death of the organism. I have demonstrated that manipulating levels of O-GlcNAc cause embryos to slow during epiboly and have dramatically altered morphology at 24hpf. I examined the role of OGT on several factors involved in epiboly such as microtubules and F-actin and found that OGT modifies the transcription factor spg possibly inactivating it.

The role of O-GlcNAc Transferase in zebrafish epiboly

I initially got interested in the role of O-GlcNAc in zebrafish embryogenesis because it had previously been shown to be modified by the transcription factor β-catenin (Zhu et al., 2001). Studies also demonstrated a role for OGT during Xenopus oogenesis as increasing O-GlcNAc in Xenopus led to O-GlcNacylation of β-catenin (Lefebvre et al., 2004). I decided to investigate if there was a role for OGT in zebrafish embryogenesis. β-catenin is dorsally accumulated in the zebrafish embryo. It is a maternal determinant of the dorsal axis. Loss of maternal β-catenin results in the disruption of dorsal specific genes. Loss of β-catenin function results in embryos exhibiting only ventral derivatives (Scheir, 2003; Scheir and Talbot 2005). Initially when I overexpressed OGT I expected to see a ventralization of embryos because I predicted O-GlcNacylation would block β-catenin function. Surprisingly, this phenotype was not observed. I instead got a heterogenous phenotype with embryos with severe body axis defects. It is possible that OGT does in fact modify β-catenin in vivo, however, it is possible the experiments performed was not enough to determine the interaction.
I found the embryos overexpressing OGT were delayed during epiboly. This led to me investigate other possible targets of OGT other than β-catenin. It had been shown that OGT modified many cytoskeletal associated proteins such as tau, and plakoglobin, as well as α-tubulin and E-cadherin (Arnold et al., 1996; Hatsell et al., 2003; Walgren et al., 2002; Zhu et al., 2001).

There are three factors that drive epiboly (Rohde and Heisenberg 2007). The first is the regulation of cell-cell adhesion by E-cadherin within the blastomeres drive epiboly (Solnica-Krezel and Cooper, 2002; Takeichi, 1991). Depleting E-cadherin in the zebrafish leads to defects in cell-adhesion as cells of the blastula are not compacted on top of the yolk and are subsequently shed from the embryo (Babb and Marrs, 2004).

Other factors such as the involvement cytoskeleton within in the yolk also play a role in epiboly (Solnica-Krezel and Driever, 1994; Betchakeu and Trinkaus, 1978). Yolk microtubules are the second factor involved in driving epiboly. The disruption of yolk microtubules during gastrulation result in epiboly defects within the germ layers during gastrulation. Also yolk microtubule disruption leads to a delay in epiboly (Cheng et al., 2004; Solnica-Krezel and Driever, 1994). The third factor to drive epiboly is the presence of actin within the YSL (Koppen et al., 2006). The presence of an actin ring within the YSL promotes contraction of the EVL epiboly (Koppen et al., 2006). Loss of this F-actin ring results in a delay in zebrafish epiboly (Wilkins et al., 2008).

Overexpressing OGT in zebrafish embryos led to a decrease in cell-cell adhesion, consistent with data demonstrating O-GlcNAcylation of E-cadherin leads to a decrease of the trafficking of E-cadherin to the cell membrane (Zhu et al., 2001).

**spg as a possible target for O-linked glycosylation to regulate endoderm formation**

Zebrafish fate mapping shows endodermal progenitors to derive from cells closest to the margin (Kimmel et al., 1990). Several signaling pathways are involved in the formation of endoderm such as the BMP, Nodal and FGF signaling pathways. More recently the spiel-ohne-
grezen (spg) gene, which was originally identified as a brain mutant, has been shown to have defects in endoderm formation (Lunde et al., 2004; Reim et al., 2004). In this study I also demonstrated that manipulating levels of O-GlcNAc lead to a reduction in endoderm. These embryos show a marked reduction in precursor endodermal cells shown by reduced axial and sox17 expression. These embryos resemble the MZspg embryos that also have a reduction in endoderm formation (Reim et al., 2004). Overexpressing OGT may lead to the inactivation of spg resulting in a loss of endoderm formation. Also, spg mutants have similar morphological defects as they are delayed in epiboly. They also exhibit a phenotype of stretched somites shown by myod expression, and the notochord of spg mutants does not extend along the dorsal axis (Reim et al., 1996). These morphological defects are similar to those observed in embryos with manipulated O-GlcNAc levels. This proposes spg as a possible target of OGT. Investigating the interaction of OGT and spg using immunoprecipitation and western blot can test this idea.

There are several other similarities between OGT overexpressing embryos and MZspg mutants. The cytoskeleton of both OGT and MZspg embryos has defects in cell shape. In this case OGT could regulate spg or factors such as E-cadherin.

All of the OGT overexpressing phenotypes are not seen in MZspg mutants. This is understandable as OGT modifies many targets. Some of the phenotypes exhibited by OGT overexpressing embryos could be a result of its modification of other targets. For example, OGT overexpressing embryos have defects in cell proliferation that spg mutants do not have.

Recently O-GlcNAc has been defined to the insulin pathway in mice (Dentin et al., 2008). When OGT is overexpressed mice become diabetic. When OGlcNAcase is overexpressed glucose homeostasis is improved (Dentin et al., 2008). This directly links O-GlcNAc to a specific pathway in animals. This data along with our findings show that O-GlcNAc does modify targets that directly link to specific pathways during animal development.

The study of O-GlcNAc is complicated because it is a ubiquitous modification. There are many targets on OGT, only a few have been discussed here. This study has presented the zebrafish as an alternative tool to study the role of OGT. These findings set precedence for the
role of OGT in endoderm formation in zebrafish. It also establishes a role for OGT in zebrafish epiboly. These are a few targets that OGT modifies and it only touches the surface of their role in zebrafish development. With zebrafish as a tool it will be possible to understand the role of OGT and its regulation in zebrafish embryogenesis.
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APPENDIX A

THE POSTTRANSLATIONAL MODIFICATION O-GLCNAC PLAYS A ROLE DURING VERTEBRATE DEVELOPMENT
ABSTRACT

The role of post-translational modifications such as phosphorylation and proteolysis in regulating the activity of developmental pathways has been studied extensively. Little is known about the role of the common post-translation modification O-linked β-N-acetylglucosamine (O-GlcNAc). O-GlcNAc occurs on serine/threonine residues of nucleocytoplasmic proteins such as cytoskeletal proteins, tumor suppressors and transcription factors and could potentially regulate multiple developmental pathways. Although studied extensively in plants and mammalian cell culture, the role of O-GlcNAc in animal development is not fully understood. The addition and removal of this modification occurs through the highly conserved enzymes O-GlcNAc Transferase (OGT) and O-GlcNAcase, respectively. I examined the role of OGT during zebrafish embryogenesis. I obtained the rat OGT and the human O-GlcNAcase and cloned them into the expression vector pCS2. I transcribed synthetic mRNA and examined the role of OGT by overexpressing OGT and O-GlcNAcase. My data indicates that changes in the levels of OGT and O-GlcNAcase lead to defects in embryonic patterning. Finally, I examined if these defects are due to developmental changes in cell fate, cell division, cell viability or morphogenesis.

INTRODUCTION

First discovered in 1984 by Torres and Hart, O-linked N-acetylglucosamine (O-GlcNAc) is a ubiquitous modification located on the serine and threonine residues of a variety of nuclear and cytoplasmic proteins. These proteins range from many transcription factors, oncogenes, viral proteins, cytoskeletal proteins and tumor supressors (Torres and Hart, 1984; Hanover et al.,
Unlike N-linked glycosylation the cycling of O-GlcNAc occurs by its addition onto a protein by a single enzyme O-GlcNAc Transferase (OGT). The removal of O-GlcNAc occurs by a single β-N-acetylglucosaminidase (O-GlcNAcase). These enzymes are analogous to protein phosphorylation and dephosphorylation catalyzed by kinases and phosphotases (Hart, 1997). This relationship suggests a “ying-yang” relationship between O-GlcNAcylation and phosphorylation (Comer and Hart, 2000).

OGT was originally identified out of extracts from rat liver in 1992 (Haltiwanger et al., 1992). The human, *C. elegans*, and zebrafish OGT were identified later (Kreppel et al., 1997; Lubas et al., 1997; Sohn and Do 2005). Each contains a tetraticopeptide repeat domain within their N-terminal domain, a catalytic C-terminal domain and are highly conserved.

O-GlcNAcase was originally purified from rat spleen in 1994 (Dong and Hart, 1994). It was later purified from bovine brain and the human gene was cloned using peptide sequencing from human brain (Gao et al., 2001). It is a bifunctional enzyme with not only O-GlcNAcase activity, but also histone acetyltransferase activity (Toleman et al., 2004). Its N-terminal domain functions as the O-GlcNAcase domain, while its C-terminal domain functions to promote eukaryotic gene transcription by increasing the acetylation of histones (Toleman et al., 2004). This led O-GlcNAcase to also be known as nuclear cytoplasmic O-GlcNAcase and acetyl transferase (NCOAT) (Toleman et al., 2004).
I wanted to see preliminary effects of rat OGT and human O-GlcNAc on zebras. I obtained the rat OGT and human O-GlcNAc from Lance Wells (UGA) and overexpressed both enzymes to determine if there was a role for O-GlcNAc in zebrafish embryos. I observed embryos with reduced heads, a shortened body axis and reduced notochords when O-GlcNAc levels were manipulated in early embryos. I conclude that manipulating levels of O-GlcNAc leads to severe morphological defects. I further investigated what could be causing these defects. These results encouraged us to clone the zebrafish OGT and further characterize its effect on early embryogenesis.

RESULTS

Altering levels of O-GlcNAc affects the development of the early zebrafish

In order to study the role O-GlcNAc plays in development, I examined the function of the enzymes that catalyze the addition and removal of O-GlcNAc. To do this I overexpressed rat OGT injecting the mRNA encoding the enzyme into an embryo at the 1-4 cell stage. I also injected the human O-GlcNacase mRNA into an embryo at the 1-4 cell stage. When overexpressing mRNA I inject before the 8 cell stage since the cells are interconnected with the yolk (Kimmel et al., 1995). This allows the mRNA to be expressed in all the cells. After the 8 cell stage a membrane forms between the cells and the yolk and they are no longer interconnected (Kimmel et al, 1995).
To determine the optimal concentration to get morphological defects I injected 100pg, 250 pg or 500pg rat OGT mRNA into embryos at the 1-4 cell stage and examined at 24 hpf for morphological defects (Kreppel et al., 1997). Expressing the enzyme at 500pg gave the greatest penetrance with approximately 30% (N=11/37) of the embryos showing defects exhibiting a phenotype of a short body axis, reduced heads, reduced hearts and reduced eyes (Figure A.1C,D).

To determine the optimal concentration to get morphological defects I injected 100pg, 250 pg or 500pg of human O-GlcNAcase mRNA into embryos at the 1-4 cell stage and examined at 24hpf for morphological defects (Gao et al., 2001). Expressing the enzyme at 500pg gave the greatest penetrance with approximately 33% (N=34/104) of the embryos exhibiting a phenotype with a short body axis, reduced heads and reduced eyes. (Figure A.1E,F).

In order to determine which tissues are affected I examined whether an increase in OGT or O-GlcNacase altered gene expression. First I examined the hatching gland, which is marked by the expression of \textit{hgg1}, which is derived from the prechordal plate (Vogel and Gerster, 2000). At 12.5hpf \textit{hgg1} expression was reduced in 38% (N=5/13) of embryos when OGT mRNA was expressed (Compare Figure A.2A and A.2B). There were fewer \textit{hgg1} expressing cells in 38% (N=12/32) of embryos injected with OGT at 18hpf (Compare Figure A.2D and A.2E). At 12.5 hpf \textit{hgg1} expression was also reduced in embryos injected with O- GlcNAcase (Compare Figure A.2A and A.2C). There were also fewer \textit{hgg1} expressing cells in embryos injected with O-GlcNAcase at 18hpf (Compare Figure A.2D and A.2F). This reduction could be a result of cell death, the cells not dividing, the cells not migrating or the fate of the cells changing.
To determine whether the defects were caused by a change in cell fate, cell differentiation, or an issue with morphogenesis, I examined the embryos at 5hpf. At 5hpf the fate of cells have been determined so if there are changes in gene expression this early in development it could indicate a change in cell fate. To determine if prechordal plate specification was affected embryos were fixed at 5hpf and also examined for the molecular marker *gooscioid* (*gsc*) (Stachel et al., 1993). *gsc* is expressed in the prechordal plate and marks the shield. At 40% epiboly *gsc* expression is normal (Figure A.3E). This implies that *gsc* expression is normal at early stages, suggesting a problem with differentiation of hatching gland, or increased apoptosis.

To test if mesoderm is affected embryos were also fixed at 5hpf and *no-tail* (*ntl*) which is expressed in the entire mesoderm and endoderm was also examined (Schulte-Merker et al., 1994). I observed gaps in *ntl* expression in 24% (N=9/37) of embryos (Figure A.3B). To further examine the reduction of mesoderm in embryos injected with rat OGT I looked at another mesodermal marker *flh*, which marks the notochord. At 50% epiboly embryos showed no change in *flh* expression as compared to control embryos (Figure A.5F). This suggests precursor notochord cells are specified.

I examine embryos later using another mesodermal derivative, *myod*, which marks the somites, (Weinberg et al., 1996). I injected 500pg of rat OGT and examined embryos at 12 hpf. 60% (N=9/15) of embryos expressing *myod* had irregular patterning of their somites as somites were stretched and elongated. This suggested that there might be an issue with morphogenesis (Figure A.5J).
Next I decided to test if endoderm is affected. I addressed this question by examining for precursor endodermal cells using the marker *sox17* (Alexander and Stainier, 1999). I examined embryos at 8hpf and showed that expressing rat OGT causes a reduction in the number of precursor endodermal cells and the for runner cells located dorsally were also reduced in 76% (N=10/13) of embryos as compared to the control (Figure A.6A,B).

Rat OGT embryos have reduced heads, so I wanted to determine of neuroectoderm fates were affected. I addressed this question by examining for expansion of neural ectoderm using markers such as *cyp26*, which marks the neural plate (Figure A.5D). I injected 500pg of rat OGT and examined embryos at 80% epiboly. 57% (N=12/21) of embryos expressing *cyp26* showed irregular *cyp26* patterning with cells expressing *cyp26* spreading away from the dorsal ectoderm (Fig A.6E).

Since these embryos have a phenotype reduced heads I examined markers such as *otx1*, which marks the rostral brain (Krauss ey al., 1992; Mercier et al, 1995). Embryos expressing *otx1* had no change in their *otx1* expression (Figure A.6H). This suggests rat OGT does not affect specification of the anterior forebrain.

In the brain *pax2.1* marks the midbrain-hindbrain boundary and otic vesicles (White et al., 1996; Krauss et al., 1992). At 12hpf 33% (N=8/24) embryos showed irregular *pax2.1* expression as the otic vesicles were arranged in an altered position as compared to control embryos (Figure A.6K). Embryos expressing *pax2.1* still expressed the gene in the midbrain-
hindbrain boundary, however the adjacent cells expressing *pax2.1* were not in their correct orientation.

**Overexpressing the N-terminal rOGT causes morphological defects**

Another approach to studying the role OGT may play in development is to inhibit OGT by knocking down its function using a dominant negative construct. OGT acts as a homotrimer. It mediates its interactions through its N-terminal domain. Expressing a N-terminal OGT (N-OGT) will prevent formation of active trimers, inhibiting enzyme activity. To perform this experiment I overexpressed the rat N-OGT in wild type zebrafish embryos. I injected 500pg mRNA of rat N-OGT into wild type embryos at the 1-4 cell stage. I allowed these embryos to develop to 24 hpf and examined them for any morphological defects. At 24hpf 45% of the embryos injected with rat N-OGT exhibited a short body axis phenotype similar to embryos overexpressing rat OGT (Figure 4B). Rat OGT and rat N-OGT giving similar phenotypes was surprising, however it was shown previously that expressing the N-terminal *spy* in *Arabadopsis* gave a similar phenotype to expressing the full length *spy* construct (Filardo and Swain, 2003). I conclude that manipulating O-GlcNAc levels does affect the development of the embryos.

To test whether N-OGT causes the same defects as observed expressing full length OGT, I examined marker gene expression. I investigated if there was a defect in mesoderm when manipulating O-GlcNAc levels using the rat N-OGT further by examining markers such as *ntl*. There was a gap in *ntl* expression in 23% (N=6/30) of embryos injected with rat N-OGT (Figure A.5B). This result is similar to that obtained when expressing the rat OGT. I also examined
dorsal mesoderm using the marker gsc and found gsc expression remained normal (Figure 5D). This is also similar to the results obtained when expressing rat OGT. I also examined flh expression, which marks the notochord. I could not detect any changes in flh expression as compared to control embryos (Figure A.5F,G). Thus notochord formation is not affected by expressing either full length or only the N-terminus OGT.

I examine embryos later using another mesodermal derivative, myod which marks the somites, (Weinberg et al., 1996). I injected 500pg of rat N-OGT and examined embryos at 14 hpf. 57% (N=10/17) of embryos had stretched and irregular patterning of their somites, similar to embryos that were expressing rat OGT (Figure A.5J).

I also examined embryos for changes in distrubution of precursor endodermal cells using the marker sox17 (Alexander and Stainier, 1999). At 8hpf rat N-OGT caused a reduction in the number of precursor endodermal cells and the dorsal fore runner cells in 70% (N=7/10) of embryos (Figure A.6A,B). This result is similar to embryos overexpressing rat OGT.

I next asked if the reduction of mesoderm was accompanied by an expansion of ectoderm using the marker cyp26. I injected 500pg of rat NOGT and examined embryos at 80% epiboly. 40% (N=6/15) of embryos expressing cyp26 showed irregular cyp26 patterning with cells expressing cyp26 spreading away from the dorsal ectoderm (Figure 6F).

Since these embryos expressing rat N-OGT also have reduced heads I examined markers such as otx1 (Krauss ey al., 1992; Mercier et al, 1995). There was no change in otx1 expression...
This suggests that the brain is still specified, even though there is a reduction in the head. This result is similar to the results expressing the full length OGT.

I examined embryos using the marker *pax2.1* at 12hpf (White et al., 1996; Krauss et al., 1992). 36% (N=9/25) of embryos expressing *pax2.1* also showed irregular *pax2.1* expression (Figure A.6L). Embryos expressing *pax2.1* still expressed the gene in the midbrain-hindbrain boundary, however the adjacent cells expressing *pax2.1* were not in their correct orientation.

**DISCUSSION**

To study if O-GlcNAc plays a role in early zebrafish development I manipulated levels of o-GlcNAc using the rat OGT and the human O-GlcNAcase. I found that overexpressing the mRNA of OGT into a recently fertilized wild type embryo does cause morphological defects in embryonic patterning. The same is true when O-GlcNAcase is overexpressed. When examined morphologically the embryos exhibited a mixed phenotype.

With only about 30% of the embryos exhibiting a phenotype in live embryos at 24hpf the overall penetrance is low. One explanation for this low penetrance could be a result cross species overexpression, injecting rat and human enzymes into a zebrafish. Since OGT is a regulated gene, another possible explanation for the low penetrance could be that there is an endogenous regulator that cannot be overcome. Therefore, even if OGT is overexpressed only the required amount of OGT may be translated. Another explanation for only 30% of embryos exhibiting a phenotype at 24hpf could be as a result of 35% of embryos dying during late gastrulation.
These results conclude that expressing rat and human enzymes cause a defect in zebrafish embryogenesis. These results encourage us to clone the zebrafish OGT and characterize its function in vivo.

**Manipulating O-GlcNAc levels causes a disruption in morphogenesis during gastrulation**

I then examined embryos earlier during development to see if the defects caused by rat OGT are due to cell death, and issue with cell fate, or an issue with morphogenesis. I examined embryos at 12hpf and 18hpf looking at hatching gland expression. I show that the hatching gland expressing cells were not patterned in a necklace around the head at 12hpf and there are few hatching gland expressing cells at 18hpf. I could not conclude if this was a result of cell death, a change in cell fate or an issue with morphogenesis so I examined embryos earlier.

At 5hpf I show that precursor mesodermal cells are specified as there was *ntl*, *gsc* and *flh*. However, later during gastrulation at about 14hpf, I show that somites are specified using the myod probe but their positions had been altered and they had become stretched and elongated. This led us to believe there was a morphogenesis issue during late gastrulation.

I also examined ectodermal precursor cells as also show that they had been specified but their position altered. I do not see defects in the specification of mesoderm, endoderm or ectoderm early. This suggests that manipulating levels of O-GlcNAc do not alter the specification of cells. However, these cells are not arranged in their correct position in the embryo. This led us to
believe that manipulating O-GlcNAc levels causes a disruption in morphogenesis during gastrulation.

Expressing both the rat OGT and rat N-OGT leads to similar defects with reduced heads and a shortened body axis. One explanation for this is expressing the full length OGT caused a reduction in OGT activity. Expressing the human O-GlcNAcase also gives a similar result of reduced heads and a shortened body axis. This could indicate that expressing the full length rat OGT could in fact lead to a reduction in OGT activity.

**Manipulating levels of O-GlcNAc causes a reduction in endoderm**

Zebrfish fate mapping shows endodermal progenitors to derive from cells closest to the margin (Kimmel et al., 1990). The commitment of endodermal cells begins just after the onset of gastrulation (Ho and Kimmel, 1993). Several signaling pathways such as Nodal, FGF and BMP signaling pathways have been shown to be crucial players in the formation of endoderm. More recently the *speil- on- grezen* (*spg*) gene, which had been originally identified as a brain mutant, has been shown have defects in endoderm formation (Lunde et al, 2004; Reim et al., 2004). I overexpressed the rat OGT and found that it caused a reduction in precursor endodermal cells. These embryos resemble the *MZspg* which also show a reduction in endoderm. I also overexpressed the N terminal rat OGT and found the same result. Similarly embryos expressing rat OGT leads to a reduction in precursor endodermal cells indicating manipulating levels of O-GlcNAc may play a role in endoderm formation.
CONCLUSION

I show that we can manipulate O-GlcNAc levels in the zebrafish using human OGA and rat OGT. These findings show manipulating levels of O-GlcNAc play a role during zebrafish embryogenesis. To better understand the role of O-GlcNAc in zebrafish embryogenesis we decided to identify and characterize the zebrafish OGT. I will manipulate levels of OGT using the zebrafish homolog and identify what role it might play.

MATERIALS AND METHODS

Subcloning rat OGT, rat NOGT and human O-GlcNAcase

I sub-cloned rat OGT, rat N-OGT, human OGA gifts from Lance Wells, into an expression vector for transcription pCS2. I then transcribed sense RNA using the Ambion RNA transcription kit

Microinjections

500 pg mRNA of rat OGT, human OGA and rat NOGT was injected at the 1-2 cell stage of wild type embryos and embryos allowed to develop and fixed at appropriate stages for in situ hybridization.
Whole mount in situ hybridization

Embryos will be fixed and processed for in situ hybridization. Antisense probes were transcribed using the Roche DIG Labeling kit. Preparation for in situ hybridization was carried out by fixing embryos overnight with 4% PFA at the appropriate stages. Embryos are manually dechorinated and dehydrated in methanol (MeOH). in situ was carried out by rehydrating embryos by washing in a series of MeOH:PBT. This is followed by nine washes of 100% PBT. Embryos are incubated at 70°C in hybridization buffer (hyb) for 2 hours and then the appropriate probe overnight. Embryos are then washed in a series of hyb/2XSSC, followed by a series of 0.2XSSC/PBT. Embryos are blocked for 2 hours in 2mg/ml BSA and incubated overnight in a 1:2000 dilution of pre-absorbed anti-dig antibody in 2mg/ml BSA. Embryos are washed the following day in 2mg/ml BSA in PBT and equilibrated in NTMT. Embryos are then stained with NBT/BCIP. After staining the reaction is stopped with 4% PFA and embryos stored at -20°C in 100% MeOH.

I used the following probes: **gsc** (Stachel et. al., 1993), **flh** (Talbot et al., 1995), **ntl** (Schulte-Merker et al., 1994) **myod** (Weinberg et al., 1996), **hgg1** (Vogel and Gerster, 2000), **mezzo** (Poulain and Lepage, 2002), **sox17** (Alexander and Stainier, 1999), **emx1** (Morita et al., 1995), **krox20** (Oxtoby and Jowett, 1993), **pax2.1** (Krauss et al., 1992).
REFERENCES


acetylglucosamine:polypeptide beta-N-acetylglucosaminyltransferase."


Figure A.1. Overexpressing the enzyme responsible for O-GlcNAc modification causes morphological defects. Embryos were injected with 500pg/ml mRNA. A. β-gal injected lateral view; B. β-gal injected ventral view; C. rat OGT injected lateral view; D. rat OGT injected ventral view; E. human O-GlcNAcase injected lateral view; F. O-GlcNAcase injected ventral view
Figure A.2. *hgg1* expression is altered when OGT is overexpressed in embryos. Embryos were injected with 500pg/nl mRNA A. β-gal injected at 12hpf; B. rat OGT injected at 12hpf; C. human O-GlcNacase injected at 12hpf D. β-gal inj at 18hpf; E. rat OGT injected at 18hpf; F. human O-GlcNacase injected at 18hpf.
Figure A.3. Mesoderm is still specified when OGT is overexpressed. Embryos were injected with 500pg mRNA. *in situ* hybridization for *ntl* (A-C) animal view, and *gsc* (D-F) dorsal view. Arrows point to gap.
Figure A.4. Expressing rNOGT causes morphological defects similar to embryos expressing rOGT. Embryos were injected with 500pg mRNA. A. β-gal injected at 24hpf B. rat NOGT injected at 24hpf.
Figure A.5. Manipulating levels of O-GlcNAc causes defects during gastrulation. Embryos were injected with 500 pg mRNA. β-gal injected at 5 hpf (A, C, E). β-gal injected at 14 hpf (H). Embryos were also injected with rat OGT and fixed for in situ. Mesodermal precursor cells are specified early (B, D, F, G). During gastrulation mesoderm is also specified, however patterning is disrupted (I, J).
Figure A.6. Altering levels of O-GlcNAc leads to a reduction in endoderm, but not ectoderm. Embryos were injected with 500pg mRNA. β-gal injected at 8hpf (A,D), 12hpf (G), and 14hpf (J). Endodermal precursors cells are reduced when levels of O-GLcNAc are manipulated (B,C). Ectodermal derivatives are specified, however their position is altered (E,F,H,I,K,L).
APPENDIX B

THE ROLE OF V.2 ZEBRAFISH O-GLCNAC TRANSFERASE IN EMBRYOGENESIS
ABSTRACT

O-GlcNAc is a ubiquitous regulatory post-translational modification on the serine and threonine residues of nucleocytoplasmic proteins. It modifies a wide variety of target proteins including cytokeletal proteins, tumor suppressors and transcription factors. Although studied extensively in plants and mammalian cell culture, the role of O-GlcNAc in animal development is not understood. Unlike other post-translational modifications that are restricted to the Golgi and Endoplasmic Reticulum, O-GlcNAc modification of nucleocytoplamic proteins is reversible, suggesting that it may regulate the activity of target proteins. The addition and removal of this modification occurs through the highly conserved enzymes O-GlcNAc Transferase (OGT) and O-GlcNAcase respectively. Zebrafish has two ogt genes, which encode several isoforms of the protein. I cloned a transcript encoding the variant 2 isoform of OGT from a 15-19hpf cDNA library. Embryos overexpressing or lacking the enzyme are slowed during epiboly and have dramatically altered morphology at 24hpf. I detect no differences in specification of mesoderm or ectoderm in these embryos. I conclude that O-GlcNAc modification regulates the activity of some protein or proteins involved in morphogenesis and is also involved in the specification of endoderm.

INTRODUCTION

O-GlcNac Transferase (OGT) is a ubiquitous enzyme responsible for catalyzing the attachment of O-GlcNAc to nuclear and cytoplasmic proteins. It is highly conserved from C. elegans to humans (Hart, 2007). The role of O-GlcNAc Tranferase has been studied extensively
in cell culture and plants. In *Arabidopsis* OGT is encoded by two genes, *spindly (spy)* and *secret agent (SEC)*. *spy* and *sec* play a role in regulating the Gibberellin (GA) pathway (Jacobsen et al., 1996). GA is a plant hormone required during most stages of plant development (Hedden and Phillips, 2000). A plant deficient in GA will exhibit a dwarfed phenotype with shortened leaves, stalks and flowers (Hedden and Phillips, 2000). Depletion of *spy* leads to a plants exhibiting a stretched, elongated phenotype. This shows there is a developmental significance of OGT in the plant.

The *spy:sec* double mutant lacks all OGT function and is embryonic lethal showing that the enzyme is essential for cell viability. There is a similar defect when OGT is depleted in mammals. The OGT gene is on the X- chromosome of in mouse and human (Shafi et al., 2002; Nolte et al., 2002). Deletion of OGT in mouse embryonic stem cells (ES cells) results in the failure of the ES cells to divide resulting in cell death (Shafi et al., 2000). Since it is difficult to generate an animal knock out for OGT there has been little study on its role in the animal in vivo.

Zebrafish has been used widely as an alternative tool to study the function of many genes. I decided to identify the zebrafish OGT (zOGT). The zebrafish OGT (zOGT) sequence was then identified and it was shown that there were two copies of zOGT, like *Arabidopsis*, found on chromosome 14 designated Copy I and Copy II (Sohn and Do 2005). There are a total of six transcriptional variants generated by alternative splicing, four transcribed from Copy I and two transcribed from copy II (Figure B.1; Sohn and Do, 2005). Variant 1 and Variant 2 contains a specific exon 19, generated by alternative splicing. This introduced an additional unique to
zOGT 16 amino acid insertion (Sohn and Do, 2005). Since the full length isoform was inactive in in vitro assay, it was proposed that this domain disrupts enzyme activity.

I previously discussed the role of v.4 zOGT. Here I demonstrate that the full length isoform called v.2 zOGT, also is functional in vivo. It has previously been showed that variant 2 of zOGT had no OGT activity in-vitro (Sohn and Do, 2005). I conclude that there is a role for v.2 zOGT since overexpressing it in- vivo results in similar defects as overexpressing variant 4 of zOGT. Therefore this protein may has activity that is not detected in in-vitro assays.

RESULTS

Overexpressing v.2 zOGT results in similar defects as overexpressing v.4 zOGT

To examine the function of zebrafish OGT I cloned variant 2 out of a 15-19hpf cDNA library acquired from Bruce Appel at Vanderbilt University. I injected 500pg of variant 2 OGT mRNA into embryos at the 1-4 cell stages and examined their phenotype at 24hpf. Surprisingly, 27% of embryos die before 24hpf (N=38/142). I classified the survivors into three groups. Class I embryos exhibited a mild phenotype of reduced heads, notochords, and a short body axis (Figure B.2B; 20%, N=29/142). Class II embryos had a more severe phenotype consisting of greatly reduced heads, and no distinguishable body axis (Figure 2C; 25%, N=35/142). Class III embryos were indistinguishable from control embryos injected with bgal (data not shown; 28%, N=40/142). Embryos injected with lower doses of OGT showed no phenotype. These phenotypes occurred at a similar frequency to those embryos injected with variant 4.
Since of the reduction in the head size, I examined embryos using molecular markers specifying neural ectoderm to see if the brain was normally patterned. I injected 500pg of v.2 zOGT and fixed embryos at 24hpf. Initially I looked at the molecular marker *emx1*, which marks the forebrain (Morita et al., 1995; Figure B.3A). *emx1* is expressed normally when v.2 zOGT is overexpressed (Figure B.3H,O; N=15). I also looked to see if the pineal gland had been specified using the marker *flh* (Figure B.3B; N=15). There is *flh* expression in the brain when v.2 zOGT is overexpressed (Figure B.3I,P; N=15). In the hindbrain rhombomeres 5 and 7 were also examined using neural marker *krox20* (Figure B.3C; N=15). Rhombomeres 5 and 7 are still present by expression of *krox20* (Figure B.3J,Q). I also examined the midbrain-hindbrain boundary using the neural marker *pax2.1* (Figure B.3D). *pax2.1* was disrupted in the midbrain-hindbrain boundary however, the position of the otic vesicles had been altered as compared to the control in Class I embryos (Figure B.3K; 47%, N=7/15). In Class II embryos the midbrain-hindbrain boundary is in a different location because the overall head morphology is different (Figure B.3R; 20%, N=3/15). I conclude although the head had been reduced when v.2 zOGT was overexpressed, the forebrain, pineal gland and midbrain-hindbrain boundary are normal, though slightly altered in position. This indicates that OGT expression does not affect brain development, but possibly morphogenesis.

**Overexpressing zOGT led to a reduction in endoderm**
I then asked if endodermal cell types were affected by increasing levels of zOGT. I fixed embryos at 8hpf and examined them for expression of precursor endodermal cells of the endodermal markers *sox17* and *axial* (Alexander and Stainier, 1999; Strahle et al., 1993; Figure B.3E,F). There was a pronounced reduction in endoderm when levels of zOGT were depleted (Figure B.2L,M,S,T). This suggested that increasing zOGT activity could have an effect on the formation of endoderm.

**Mesoderm is still specified when O-GlcNAc levels are altered**

I then looked at mesodermal derivatives. The mesodermal marker *hatching gland (hgg1)* marks the hatching gland which is a derivative of the prechordal plate (Vogel and Gerster, 2000). At 12hpf *hgg1* is expressed in a reduced pattern. The *hgg1* does not extend as a necklace around the head of the embryo as compared to control embryos (Figure B.3G; 60%, N=9/15). This indicates that prechordal plate cells are still present, however their expression is altered.

**Overexpressing zOGT results in defects during gastrulation**

I then asked if mesoderm derivatives were formed properly. Initially I examined the mesodermal marker *ntl*, which marks the notochord at 10hpf (Figure B.4A). I show that the notochord does not extend completely along the dorsal axis in both Class I and Class II embryos (Figure B.4 B,C; 47%, N=7/15). I also examined the mesodermal marker *myod*, which marks the somites at 14hpf (Figure B.4D). In embryos overexpressing v.2 ZOGT somites are disorganized and stretched along the dorsal axis in Class I embryos (Figure B.4E; 47%, N=7/15). In Class II embryos somites are also disorganized however some somites are missing (Figure B.4F; 40%, N=6). In the mesoderm *pax2.1* marks the pronephros at 14hpf as a loop of expression in the
intermediate mesoderm (Krauss et al., 1992; Figure B.4G). \textit{pax2.1} expression is stretched and widened along the dorsal axis reflecting the altered shape of the body axis as compared to control embryos (Figure B.4H,I; 47%, N=7/15). To determine of somites differentiate properly I examined \textit{α-tropomyosin}, which marks the somites at 24hpf (Ohara et al., 1993; Figure B.4J). I found that the somites are highly disorganized and patterned irregularly along the trunk and tail of the embryo (Figure B.4K,L; 53%, N=8/15). I conclude that the disorganized mesoderm suggests problems with convergence and extension.

I decided to look earlier to determine what might be causing this disruption during morphogenesis. I examined live embryos over a time period of 3hpf to 12hpf. Embryos overexpressing OGT were delayed during epiboly after about 7hpf (Figure B.5). They also exhibited an aggregation of cells on the dorsal side of the embryo suggesting defects in extension (Figure B.5F,G arrowhead; 38%, N=23/60). I conclude that embryos are delayed during epiboly resulting in embryos with reduced heads and shortened body axis at 24hpf. This data is also consistent with results of overexpressing v.4 zOGT.

\textbf{Disruption in cell-cell adhesion leads to a delay in epiboly}

Cell-cell adhesion has been implicated as one factor involved during epiboly. One cause of the defects that I have shown could be a result of a decrease in cell-cell adhesion. \textit{E-cadherin} acts in complex with \( \beta \) and \( \gamma \)-catenin, along with the intracellular actin filaments to promote cell-cell adhesion. I examined embryos at 8hpf analyzing F-actin in the cell membranes using rhodamine-phallodin (Figure B.6A). Analysis of F-actin showed that manipulating levels of zOGT results in changes in the shapes of cells of the enveloping layer in embryos as compared to control
embryos (Figure B.6A,B; 60%, N=6/10). I also show that there is a decrease in cell-cell adhesion as cells fall away from the blastoderm when levels of O-GlcNAc have been manipulated as compared to control embryos (Figure B.6C,D; 60%, N=6/10). Since changes in cell shape could result from or cause changes in adhesivity, I conclude that overexpressing v.2 zOGT leads to a decrease in cell-cell adhesion.

**DISCUSSION**

**Identification and characterization of variant 2 zebrafish O-GlcNAc Transferase**

In order to study the function of OGT in the zebrafish I identified and characterized one of the variants the six variants. I cloned one 1053a.a. variant, variant 2 out of a 15-19hpf cDNA library provided to us by Bruce Appel of Vanderbuilt University. I examined the presence of neural ectoderm as there is a reduction in the head of embryos overexpressing zOGT. As shown by the neural markers *emx, flh, krox20* and *pax2.1* the brain is still patterned, as neural ectoderm derivatives are still present.

**Manipulating levels of O-GlcNAc does not affect mesoderm and ectoderm induction**

I examined the function of zOGT by depleting zOGT protein. Embryos were injected with an antisense oligonucleotide against zOGT and examined at 24hpf. Embryos were shown to have a significant reduction in the head, leading us to examine the presence of neural ectodermal cell types in these embryos. As shown by the markers *emx, flh*, and *krox 20* the brain is still patterned and present. Similarly, when the function of v. 2 zOGT was examined by
overexpression assays, embryos exhibited a similar phenotype. I also examined if the brain was patterned, and in overexpression assays there is still a presence of neural ectodermal derivatives. I show that ectodermal cell types are still present after manipulating O-GlcNAc levels.

I also examined for the presence of mesodermal progenitors. When zOGT is overexpressed I show at 12hpf the cells of the hatching gland are still specified as shown by the presence of hgg1 expressing cells. Although these cells are specified their position had been altered. Also, at 24hpf manipulating levels of O-GlcNAc causes a shortening in the body axis and reduction of the notochord. α-troposyosin marks the somites at 24hpf. Manipulating levels of O-GlcNAc causes a disruption in the expression pattern of α-troposyosin, however the somites are still present.

**Manipulating levels of O-GlcNAc regulates endoderm formation**

In this study I examine the role of zOGT during embryogenesis. I previously manipulated the levels of O-GlcNAc and showed that there were morphological defects in the embryo at 24hpf. I also showed ectodermal derivatives were present we then examined for the presence of endodermal progenitors. Several factors play a role in the formation of endoderm. One such factor is the the speil-ohen-grezen (spg) gene, which was originally identified as a brain mutant, has recently been shown to have defects in endoderm formation (Lunde et al., 2004; Reim et al., 2004). We manipulated levels of O-GlcNAc by overexpressing v.2 zOGT and look at the presence of precursor endodermal. Embryos depleted of zOGT show a marked reduction in precursor endodermal cells shown by reduced axial and sox17 expression. This results in a
phenotype similar to overexpressing v.4 zOGT. These embryos resemble the MZspg, which also show a reduction in endoderm (Reim et al., 2004). I conclude that overexpressing v.2 zOGT leads to a reduction in precursor endodermal cells indicating manipulating zOGT causes a reduction in endoderm.

**O-GlcNAc Transferase plays a role in during morphogenesis**

I examined embryos for mesodermal derivatives during gastrulation and showed that there defects during gastrulation. I show that cells of the notochord are present, however they do not fully extend along the dorsal axis and they gather at the margin in Class II embryos. I also examined the somites using the mesodermal markers myod and show that somites were specified however they exhibited a stretched and elongated phenotype. The positions of the somites were also disrupted. I examined the pronephros using the marker pax2.1 and showed that although cells were specified their positions had been altered. These results were similar to results I had observed when I overexpressed v.4 zOGT. I then decided to look earlier during development to see was causing this disruption in the position of cells.

When I overexpress v2.ZOGT I noticed a delay during the course of epiboly. Embryos would develop normally until about 5hpf when there was an aggregation of cells on the dorsal side of the embryo. Cells would continue to aggregate at the top of the yolk and migrate slower compared to control embryos. This resulted in embryos developing slower than control embryos.
I also show there was a decrease in cell-cell adhesion as embryos analyzed with rhodamine-palloidin show disorganized cells that would fall away from the blastomeres during the course of epiboly.

CONCLUSION

I have identified and characterized variant 2 of zebrafish OGT. I show that it plays a very broad role in during embryogenesis, two of which I have discussed. I conclude that OGT is required for endoderm formation. Increasing levels of O-GlcNAc lead to a reduction in endoderm providing a basis for the role of zOGT in endoderm formation. I also show that overexpressing v.2 zOGT causes delays in epiboly demonstrating its role during morphogenesis. These studies are the first to show a role for OGT in these fundamental processes. They also indicate a role for OGT in animal development and provides a basis for the study of OGT in animal development.

MATERIALS AND METHODS

Identification of endogenous zebrafish OGT

I cloned a transcript encoding the variant 2 isoform from a 15-19hpf cDNA library acquired from Bruce Appel at Vanderbilt University. I used primers obtained from the cloned sequence of the full length zOGT to identify and isolate the clone (Sohn and Do, 2005). The isolated clone was sequenced to ensure if was the correct gene of interest. Variant 2 was TOPO cloned into the XbaI site of the pCS2 plasmid.
Whole mount *in situ* hybridization

Embryos will be fixed and processed for *in situ* hybridization. Antisense probes were transcribed using the Roche DIG Labeling kit. Preparation for *in situ* hybridization was carried out by fixing embryos overnight with 4% PFA at the appropriate stages. Embryos are manually dechorinated and dehydrated in methanol (MeOH). *in situ* was carried out by rehydrating embryos by washing in a series of MeOH:PBT. This is followed by nine washes of 100% PBT. Embryos are incubated at 70°C in hybe for 2 hours and then the appropriate probe overnight. Embryos are then washed in a series of hyb/2XSSC, followed by a series of 0.2XSSC/PBT. Embryos are blocked for 2 hours in 2mg/ml BSA ad incubated overnight in a 1:2000 dilution of pre-absorbed anti-dig antibody in 2mg/ml BSA. Embryos are washed the following day in 2mg/ml BSA in PBT and equilibrated in NTMT. Embryos are then stained with NBT/BCIP. After staining the reaction is stopped with 4% PFA and embryos stored at -20°C in 100% MeOH.


**Microinjection of mRNA**

Sense mRNA was made using the mMESSAGE mMACHINE RNA synthesis kit (Ambion). I injected 500 pg of *zOGT* or β-galactosidase mRNA into chorionated embryos at the 1-4 cell
stage. Embryos were allowed to develop and examined at 24hpf or fixed at appropriate stages for
in situ hybridization.

**Labelling F-acting in fixed embryos**

At 8hpf embryos were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) overnight at 4°C. Embryos were dechroniated and washed thoroughly with PBS containing 0.1% Tween (PBT). They were incubated in blocking solution containing 2% bovine serum albumin (BSA) at room temperature for 2 hours. To label F-Actin, embryos were incubated with rhodamine-phalloidin at a dilution of 1:40 at room temperature in the dark. Embryos were then washed thoroughly in PBT. Rhodamine-phalloidin was dissolved in methanol at a concentration of 6.6µM and stored at -20°C.

**REFERENCES**


Figure B.1. Identifying zebrafish OGT. There are two copies of zebrafish OGT found on Chromosome 14. Alternative spicing generates six variants, four variants are generated from Copy I and two variants are generated from Copy II. We have cloned variant 2 from a 15-19hpf cDNA library.
Figure B.2. Manipulating levels of O-GlcNAc causes morphological defects. Embryos injected at the 1-4 cell stage with 500pg mRNA A. β-gal injected; B. Class I zOGT injected; C. Class II zOGT injected.
Figure B.3. Overexpressing OGT causes a reduction in endoderm. Embryos injected 500pg β-galactosidase and fixed at 24hpf (A-C), 14hpf (D), 8hpf (E,F) and 12hpf (G). Embryos were also injected with 500pg v.2 zOGT and fixed for in situ at 24hpf. Neural ectodermal precursor cells are specified when OGT is overexpressed (H-J, O-Q). pax2.1 expressing cells are specified but their position is altered (K,R). Embryos overexpressing zOGT causes a reduction in precursor endodermal cells (L,M,S,T). Cells of the precordal plate are specified but their position altered (N,U).
Figure B.4. OGT plays a role during morphogenesis. Embryos were injected with mRNA. β-gal injected at 10hpf (A), 14hpf (D,G) and 24hpf (J). Embryos were also injected with v.2 zOGT. Notochord is specified but does not extend along the dorsal axis (B,C). Somites are stretched and expanded (E,F,K,L). Pronopheros is specified however its position altered (H,I).
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Figure B.5. Defects during gastrulation are caused by a delay in epiboly. Embryos were injected with 500pg β-gal injected (A-D). Embryos injected with 500pg v.2 zOGT had a aggregation of cells accumulating on top of the yolk during epiboly (F,G, arrowhead). At 12hpf there was no presumptive eye (H, arrow).
Figure B.6. Overexpressing v.2 zOGT causes a decrease in cell-cell adhesion. Embryos were injected with 500pg β-gal (A,C). Embryos injected with v.2 zOGT had irregular cell shape (B) and cells of the blastoderm fall away from the yolk (D).