THE TERATOGENIC EFFECTS OF ANTI-DIABETIC DRUGS: 
*IN VITRO* ASSAYS USING EMBRYOID BODY 
MORPHOGENESIS

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Abstract

Many adults take some form of medication to treat chronic conditions, however little is known about their effects on embryogenesis and pregnancy. With the increase in fertility problems society is facing and the increase in use of medications, it is important to understand what effects pharmaceuticals have on the developing embryo to ensure that future generations are not adversely affected by teratogenic compounds that can lead to birth defects. In this study, teratogenicity of anti-diabetic compounds was examined using the in vitro P19C5 embryoid body (EB) elongation morphogenesis model. As the EB model recapitulates key embryological events crucial for body patterning and axis formation, adverse impact of drugs on EB growth and elongation implicates their potential teratogenicity. Of the 31 anti-diabetic compounds examined, 27 decreased EB size and 11 either decreased or increased EB elongation at a given concentration. Morphogenetic impact of four specific anti-diabetic drugs, dapagliflozin, phenformin HCl, manidipine 2HCl, and resveratrol, was further examined by investigating expression patterns of key developmental regulators. Each drug exhibited a unique effect on gene expression patterns. Notably, dapagliflozin significantly reduced the level of the Wnt3 gene, a crucial regulator of the primitive streak formation. The Wnt-inhibiting effect of dapagliflozin was also confirmed by the TOPFLASH reporter assay. Furthermore, it was revealed that the drug impact on EB size was likely mediated by adverse effect on cell proliferation. These results suggest that anti-diabetic compounds impact EB morphogenesis by perturbing key developmental regulators, cellular signaling, and proliferation. The present research should lay the foundation for further investigations, including animal and human studies, to determine teratogenicity of specific anti-diabetes medications.
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Chapter 1. Introduction

1.1. Environmental factors influence embryo development

Every year, approximately 7.9 million babies, or 6% of all babies in the world, are born with serious birth defects (Lobo & Zhaurova, 2008), which not only compromises the quality of many peoples’ lives but also puts a huge economical and emotional burden on society. Genetic and environmental factors can cause birth defects. Approximately 20% of all birth defects are caused by genetic factors, including chromosome abnormalities and gene mutations such as Down syndrome (trisomy 21) and Tay-Sachs disease (HEXA gene mutation), respectively. Approximately 10% are caused by environmental agents, known as teratogens (see 1.2). About 20% are caused by multifactorial influences, i.e., a combination of genetic and environmental factors. Unfortunately, approximately half of all birth defects have an unknown cause (Cleveland Clinic, 2012; Figure 1). Thus, more research efforts are required to understand the causes of all birth defects in order to minimize such tragic incidences.

For mammalian embryos, the entire process of normal development occurs in the mother’s reproductive tract, and thus the fate of the embryo is greatly influenced by maternal conditions. In humans, approximately one day after fertilization in the oviduct, the embryo reaches the 2-cell stage and moves towards the uterus via contractions and cilia movement in the oviduct. The embryonic cells, or blastomeres, divide further to become 16-32 cells and merge to form a morula by Day 4 to 5. Once a cavity forms and expands, the embryo is termed a blastocyst. By the end of the 1st week, the blastocyst hatches out of the eggshell, or zona pellucida and implants into the uterine wall. During the 2nd week of development, the embryo forms the epiblast, hypoblast, and amniotic sac. Near the beginning of the 3rd week, the
primitive streak emerges in the epiblast at the future caudal end, and gastrulation commences to generate the three germ layers: the ectoderm, mesoderm, and endoderm. A functional placenta composed of tissues from the embryo and the mother starts to form during the 3rd week, which provides oxygen, nutrients, and hormones to, as well as removes waste products from, the developing embryo. The 3rd to 8th weeks of development mark the critical period of body patterning. During this period, the foundations of the major organ systems are established. Importantly, this is when the embryo is most susceptible to environmental disturbances. Exposure to teratogens during this period has the highest chances of causing embryonic death or birth defects (Gilbert, 2014).

1.2. Identifying teratogens is challenging

Teratogens are environmental factors that negatively affect embryo development to cause birth defects. To date, various teratogens have been identified, which can be categorized into four groups: physical agents, infectious agents, chemical agents, and maternal conditions (Table 1). Physical agents include ionizing radiation, such as X-rays or gamma rays, which have been linked to microcephaly (smaller than normal head size) in infants born of mothers given pelvic irradiation during pregnancy (Murphy, 1928). Infectious agents, such as bacteria, viruses, or parasites can also harm developing embryos when the mother is infected during pregnancy. The first example of a teratogenic infectious agent discovered was the rubella (German measles) virus, which causes distinct defects in the fetus, such as congenital cataracts (Gregg, 2001). Chemical agents, such as drugs and environmental pollutants, can also be teratogenic. Prenatal exposure of alcohol can result in fetal alcohol syndrome, which is marked by growth deficiency,
facial abnormalities, and central nervous system disorders (Mattson, Schoenfeld, & Riley, 2001). Another well known chemical agent linked to birth defects is thalidomide, which was initially marketed as a sedative to treat nausea in pregnant women. Widespread use in the late 1950s and early 1960s resulted in thousands of children born with severe birth defects, such as congenital heart disease, ear and eye malformations, and most notably, shortened limbs (Kim & Scialli, 2011). Maternal conditions, such as malnutrition, diabetes, and thyroid disorders, make up the last category of teratogens. Diabetes mellitus, a group of metabolic diseases that leads to hyperglycemia, or high blood glucose, can result in a range of malformations, known as diabetic embryopathy (see 1.5).

While the definition of teratogens is seemingly simple, their natures and mechanisms of actions are diverse and complex. James G. Wilson, an embryologist and anatomist, published the Six Principles of Teratology (Wilson, 1959, 1973), stating that teratogen sensitivity depends on the genotype, developmental stages of exposure, dose, and nature of the teratogen (Wilson, 1973). Thus, a plethora of factors, including the dose, exposure time, point in pregnancy, genetic background of the embryo, and maternal conditions, such as nutrition, external environment, and access to prenatal care, can contribute to whether a certain environmental agent could cause birth defects or not. For example, a fetus may be unaffected by a certain medication during late pregnancy, but it may develop birth defects if it is exposed to the same medication at the same dose during early development, particularly when body patterning and organogenesis occurs in the 3rd to 8th weeks of pregnancy.

Due to the complex nature of teratogens, multiple investigative approaches are needed to determine teratogenicity of a certain agent. There are currently three different types of
approaches to assess the potential teratogenicity of agents: human epidemiological studies, \textit{in vivo} animal experiments, and \textit{in vitro} model studies (Table 2). Epidemiology is the study of the distribution and determinants of health-related events, and the application of the study to the control of diseases and other health problems (WHO, 2015). There are two main study designs: case control studies and cohort studies. Case control studies compare the frequency of exposure of an agent, while cohort studies examine the rate of a disease in pregnancy in groups of infants with a specified malformation to groups of infants (control) without the malformation of interest (Bertollini, Pagano, & Mastroiacovo, 1993). These studies can be effective at identifying teratogens. However, they are retrospective, i.e., definitive conclusions can only be reached after many cases of birth defects have been documented. Thus, other assessment approaches are required to predict potential teratogenicity of agents and to prevent such tragic outcomes.

\textit{In vivo} animal studies using mice, rats, rabbits, and non-human primates are currently used for preliminary evaluation of the reproductive and developmental toxicity of chemical agents. An agent is administered to pregnant animals, and incidences of embryo loss or fetal malformations are assessed. There is no single animal model that is representative of a human model because of metabolic, maternal-fetal, and genetic differences, so not all results can be extrapolated to humans. For example, thalidomide was marketed as safe for maternal consumption because it had no effect on pregnant rats and their pups, which were found to be thalidomide-resistant species. Tragically, this led to thousands of birth defects in human infants. Later studies on rabbits showed that they were sensitive to thalidomide (Hansen & Harris, 2004). Additionally, administered drugs are significantly processed by the metabolic system of
the mother, which are highly variable depending on the species. Thus, the impact of drugs on embryo development, namely exposure levels and mechanisms of teratogenic actions, may not be accurately reflected using in vivo systems. This may lead to a drug being inaccurately labeled as safe or teratogenic based on animal studies. Thus an emphasis on additional approaches is necessary to reduce such pitfalls. In addition, in vivo animal studies are time-consuming, expensive, and require the sacrifice of the animal subjects, which raises significant animal welfare concerns.

1.3. In vitro test systems for teratogen studies

The third approach to identifying a potential teratogen is through in vitro studies. There are thousands of pharmaceuticals being used and innovated, however, only a small fraction have been tested for their potential developmental toxicity, and thus, a fast and relatively cost-effective approach is needed for the initial investigation of drugs—in vitro approaches. In vitro models that have been used for teratogen tests include whole embryo culture tests, organ culture assays (e.g. micromass culture), and stem cell culture systems (e.g. embryonic stem cell test) (Schumann, 2010). These in vitro studies are advantageous for testing potential teratogens because they are cost- and time-effective, do not require as many animals to be sacrificed, simple, easy to manipulate, and most importantly, the exact dose of chemical agent exposed to the system is known because there is no maternal interference.

Whole embryo culture (WEC) tests expose potential teratogens to embryos cultured at early states of organogenesis. Mammalian (rat and mouse embryos) and non-mammalian vertebrates (Xenopus frog and zebrafish embryos) have been utilized for teratogen screening
(Schumann, 2010). For mammalian tests, rodent embryos are removed from the uterus and cultured for one or two days in medium, which allows a limited but reproducible developmental progress. Specific advantages to the WEC system include the ability to assess various developmental events, such as dynamic morphogenesis and organ formation.

Disadvantages, particularly for mammalian tests, are the number of embryos that can be prepared in a day by a person (approximately 50), the limited embryogenesis period in culture, and the relatively high cost (monetary and time-wise). While non-mammalian species are mostly devoid of these disadvantages, the translatability of results to humans is often uncertain (Webster, Brown-Woodman, & Ritchie, 1997).

Another type of *in vitro* assay for teratogen testing is the micromass culture, which was first developed by Flint in 1980 using rat limb bud cells (Brown *et al.*, 1995). Rat, mouse, or chicken embryo limb bud or central nervous system cells are dissociated to create primary cell suspensions, which are then placed on a culture dish to generate patches of cells, i.e., a micromass. The micromass cultures retain some aspects critical to embryogenesis, such as adhesion, division, differentiation, movement, and communication (Ozolins, 2009), so a chemical’s impact on these cellular events can be investigated effectively. This assay has been used to test potential reproductive and developmental toxicity of new chemicals, and studies have shown that it can distinguish between teratogens and non-teratogens within particular classes of chemicals (Brown *et al.*, 1995). The disadvantages are that not all teratogens are detected with this assay (Uphill, Wilkins, & Allen, 2002), not all results can be translatable, and animals must still be sacrificed.
The embryonic stem cell test (EST) is a type of in vitro assay developed by Spielmann et al. that utilizes two mouse cell lines, D3 embryonic stem cells (ESCs) and 3T3 fibroblasts, in order to assess the potential embryotoxicity of chemicals (Spielmann et al., 1995). There are three selected endpoints in the EST for predicting embryotoxic potential of compounds: inhibition of ES cell differentiation into cardiomyocytes and inhibition of ES cell and 3T3 cell growth (Kuske, Pulyanina, & zur Nieden, 2012). The concentrations of chemicals that affect these endpoints are incorporated into a unique prediction model, or mathematical formula, which is then used to classify potential embryotoxicity into one of three classes: strong, weak, and not embryotoxic (Genschow et al., 2002). In an initial validation study using 16 test chemicals with known embryotoxicity, the EST was able to correctly assign most of them to the 3 classes (Genschow et al., 2002; Spielmann et al., 1997). However, a follow-up validation study using an additional 13 reference compounds yielded poor performance, as only 2 of 13 were assigned correctly (Marx-Stoelting et al., 2009). Further efforts have been put forth to improve the predictive performance of ESTs, including testing of ESC differentiation into other cell types, such as neurons (Theunissen et al., 2010) and osteoblasts (de Jong, van Beek, & Piersma, 2012), and also use of human ESCs (Colleoni et al., 2011) to overcome potential inter-species differences. Importantly, however, animals do not need to be sacrificed for ESTs because only permanent cell lines are used, which is an advantage unique to this system.

1.4. Axial elongation morphogenesis of P19C5 EB as a new in vitro teratogen assay

A new type of EST utilizes the P19 mouse embryonal carcinoma stem cell line, which can be used to test the potential teratogenicity of chemicals. Previous studies demonstrated that
aggregates of P19 cells and the sub-clonal line of P19C5 cells undergo axial elongation morphogenesis in vitro (Marikawa et al., 2009; Lau & Marikawa, 2014). In hanging drop culture, these stem cells initially form into spherical aggregates, or embryoid bodies (EBs), and later transform into an elongated shape. P19C5 cells remain spherical for the first two days of culture, and they distinctly elongate by the fourth day (Figure 2), which is more rapid than the elongation of parental P19 cells. Gene expression profiles suggest that the elongation morphogenesis of P19C5 EBs represents primitive streak formation, gastrulation, and body patterning and elongation along the anterior-posterior axis, which normally takes place between E5.5 and E8.5 of mouse embryonic days. These stages roughly correspond to the 3rd to 4th weeks of human development, which overlaps with the critical period that is most sensitive to teratogens. The P19C5 EB culture has been proposed as a screening tool for chemicals to determine their potential teratogenicity. The most unique aspect of the P19C5 system, compared to other ESTs, is exploitation of dynamic and complex embryological events, i.e., morphogenesis. Morphogenesis is driven by various cellular processes, such as differentiation, proliferation, migration, adhesion, shape change, and death. Thus, in vitro recapitulation of morphogenesis represents a broader aspect of embryo development than other ESTs, which are based on differentiation of cells into a specific cell type. Various birth defects are due to abnormal morphogenesis, including neural tube closure defects, heart septal defects, and cleft lips and palates. In addition, the four day culture period to attain EB elongation is much faster than other EST methods, e.g., differentiation of detectable beating cardiomyocytes in ES cells takes about 10 days. Also, the extent of P19C5 EB elongation can be measured quantitatively based on the Elongation Distortion Index (EDI) using an image analysis software (Marikawa et
al., 2009), which allows for effective assessment of chemical impact on EB elongation morphogenesis. Thus, morphogenesis of P19C5 EBs should serve as a valuable in vitro model to investigate and screen the potential teratogenic impact of environmental agents.

1.5. Diabetes and anti-diabetes medications

A disease of particular interest that affects more than 29.1 million Americans in the U.S., is diabetes mellitus, commonly referred to as diabetes, which is a condition where a person has hyperglycemia, or higher than normal blood glucose levels (CDC, 2014). In 2014, diabetes was estimated to affect 9% of adults aged 18 and older in the entire world, and it is projected to be the seventh leading cause of death in 2030 (WHO, 2015). There are three types of diabetes: Type 1, Type 2, and gestational. Type 1 diabetes, previously known as juvenile or insulin-dependent diabetes, occurs when the body does not produce enough insulin. Insulin is a peptide hormone produced by beta-cells in the pancreas that promotes the absorption of glucose from the blood. Only 5% of diabetes cases are Type 1. Administration of insulin is typically used to treat Type 1 diabetes. Type 2 diabetes, previously known as adult-onset or non-insulin-dependent diabetes, occurs when the body develops insulin resistance in spite of sufficient production of insulin, which results in high blood glucose levels. This is the most common form of diabetes, and many different types of medications are used to treat it. Gestational diabetes is a type of diabetes that develops during pregnancy, usually around the 24th week. All pregnant women have some insulin resistance during late pregnancy. However, some women become more resistant than others, which can lead to gestational diabetes.
Diabetes increases the likelihood of congenital malformations, known as diabetic embryopathy, by at least double the rate seen in nondiabetic pregnancies. Malformations can affect multiple organ systems, including the central nervous system (spina bifida, anencephaly, holoprosencephaly), musculoskeletal (caudal regression), cardiovascular (ventricular septal defect, situs inversus), gastrointestinal (renal atresia), and genitourinary (renal agenesis), all of which can result in pre- or postnatal mortality or morbidity (Allen & Armson, 2007). A study showed that approximately 2-3% of babies born in Canada have major congenital anomalies, but this number was increased by 2- to 3-fold (4-10%) in infants of diabetic mothers (Allen & Armson, 2007). These malformations arise when diabetes affects the fetus during the 3rd to 8th weeks of pregnancy, which is the period when organogenesis occurs. Unfortunately, the mechanism by which diabetic embryopathy occurs still remains unknown. Thus, it is important to control diabetes during pregnancy. However, treatment of diabetes must be conducted in a careful manner because medications could be teratogenic themselves. For example protein kinase C (PKC) inhibitors (e.g., ruboxistaurin) are used to treat diabetic retinopathy (Shen et al., 2008), but they should not be used during pregnancy because they would disrupt angiogenesis, which is necessary for implantation, placentation, and organogenesis (Loeken, 2008).

1.6. The teratogenicity of anti-diabetes medications is largely unknown

There are different classes of drugs taken orally that work to lower blood glucose levels to restore glucose homeostasis by targeting various organs and molecules to treat diabetes, as detailed in Table 3. In essence, drugs of the sulfonylurea, meglitinides, and DPP-4 inhibitor classes target the pancreas to cause beta-cells to release more insulin, which thereby decreases
blood glucose levels. Drugs in the biguanide and thiazolidinedione classes decrease blood glucose levels by inhibiting liver gluconeogenesis and glycogenolysis, which produce glucose. Drugs of the SGLT2 inhibitor class work by decreasing the reabsorption of glucose in the kidneys, which leads to an increased excretion of glucose in the urine. Thiazolidinedione-classed drugs sensitize adipocytes and myocytes to insulin, which increases their uptake of glucose. Drugs in the alpha-glucosidase inhibitor and bile acid sequestrant classes work on the gastrointestinal system to lower blood glucose levels.

Most anti-diabetic drugs are deemed as safe for use during pregnancy or there are not enough animal or human studies conducted to contraindicate their use. The FDA categorizes most drugs according to their potential risk to cause birth defects if they are used during pregnancy. Categories are determined based on studies and the risk to benefit ratio. The FDA pregnancy risk categories are: A, B, C, D, and X. Category A and B drugs are deemed safe for use during pregnancy, while Category C drugs do not have adequate studies to contraindicate drug use during pregnancy. Category D and X drugs are contraindicated for use during pregnancy because there is evidence showing human fetal risk and abnormalities. Most anti-diabetic drugs are Category B or C drugs, and thus further studies should be conducted to ensure that they are not potentially teratogenic.

1.7. Objective of thesis project

The objective of my Master Thesis Project is to examine the potential teratogenic effects of diabetes-related drugs using the \textit{in vitro} P19C5 stem cell morphogenesis model, which mimics some critical aspects of embryogenesis, including gastrulation and axial patterning.
Experiments were designed to determine the morphogenetic impact of various diabetes-related drugs, and several selected drugs were further evaluated to understand morphogenetic impact at the molecular level. Experimental results are expected to be useful for determining what drugs should be further tested in additional in vitro and in vivo tests to confirm their teratogenicity, and they are also expected to shed light on their mechanism of teratogenic actions. My overall goal is to exploit the unique and convenient in vitro P19C5 EB morphogenesis system in order to build the foundation for future studies on the potential teratogenicity of anti-diabetic medications. Diabetes is a known teratogen, and we want to reduce the occurrences of birth defects in babies born to women with this disease. To do this, we need to know whether anti-diabetic drugs themselves are teratogenic, and this is what I strived to do in my project.
Chapter 2. Materials and Methods

2.1. Cell Culture and Embryoid Bodies (EBs)

P19C5 cells (Lau & Marikawa, 2014) were maintained in Minimum Essential Medium (MEM) α, nucleosides, GlutaMAX Supplement (Life Technologies), 7.5% newborn calf serum, 2.5% fetal bovine serum, 50 µg/ml streptomycin, and 50 units/ml penicillin. Cells were passaged every other day when more than 75% confluency was achieved in culture flask. P19C5 embryoid bodies (EBs) were generated in hanging drop culture containing 1% dimethyl sulfoxide (DMSO), which induces spontaneous P19C5 EB elongation and mesoderm formation, at a density of 10 cells/µl according to previous methods (Marikawa et al., 2009; Lau & Marikawa, 2014). Drops (20 µl each) of cell suspension were pipetted onto the inner surface of Petri dish lids. One control plate with no drug and four drug-treated plates (0.1, 1, 10, and 100 µM), each of which contained 16 hanging drops, were prepared using the same cell suspension. Plates were cultured for four days without any medium change. Morphology and integrity of EBs were observed every day, and on Day 4, EBs were collected from hanging drops and photographed. Size and Elongation Distortion Index (EDI) were measured for morphometric analysis (See 2.3).

2.2. Anti-diabetic Drugs

All 31 drugs used in this study were obtained from Selleck Chemicals’ Anti-diabetic Compound Library (Table 4). All drugs were dissolved in DMSO for a final drug concentration of 10 mM and stored at -20°C.
2.3. Morphometric Analyses

Day 4 EBs were collected from hanging drops and placed into a PBS-containing Petri dish for photography. Images were captured with an AxioCam MRm digital camera (Carl Zeiss, Thornwood, NY) attached to an Axiovert 200 inverted microscope with Hoffman modulation contrast optics (Carl Zeiss) and controlled by AxioVision software (Carl Zeiss). Size and Elongation Distortion Index (EDI) were measured for morphometric analysis. AxioVision images were converted into JPEG files and opened in ImageJ (http://imagej.nih.gov/ij/). Individual EB circumferences were traced using the polygon tool, and their area and circularity were measured. Area was used to approximate the size of EBs, and circularity was used to calculate the Elongation Distortion Index (EDI), using the equation (1/[circularity]-1), which is identical to ((perimeter)^2/4π[area]-1) (Marikawa et al., 2009).

2.4. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Day 1, 2, 3, and 4 EBs were collected and total RNA was extracted with TRIzol reagent (Life Technologies) and Direct-zol RNA MiniPrep kit (Zymo). Total RNA (300ng/ml) was processed for cDNA synthesis using M-MLV Reverse Transcriptase (Promega) and oligo-dT primer in a 25 µl reaction. Quantitative reverse transcription PCR (qRT-PCR) was performed using a CFX96 real-time PCR detection system (Bio-Rad) using SsoAdvanced SYBR Green Supermix (Bio-Rad) as follows: initial denaturation at 94°C for 5 min, followed by up to 45 cycles of 94°C for 15 sec, 60°C for 20 sec, and 72°C for 40 sec. Data files were opened using CFX Manager software (Bio-Rad) and Ct values were transferred to Microsoft Excel for further analyses. Actb was used as the housekeeping gene to normalize expression levels of other
genes. Sequences of primers used are shown in Table 5. Expression analyses were conducted using three independent sets of samples.

2.5. Cell Proliferation Assay

P19C5 cells were plated on a 24-well plate at $2 \times 10^4$ cells per well. Five wells were used for each experiment set: one control well containing 1% DMSO only and four drug-treated wells containing 0.1, 1, 10, and 100 µM drug with final concentration of 1% DMSO. After 48 hours, cells were trypsinized, fully dissociated by pipetting, and counted using a hemocytometer. Experiments were done in triplicates using three independent cell samples.

2.6. Plasmid Transfection and Dual-Luciferase Assay

A day before transfection, $2 \times 10^4$ P19C5 cells were plated per well in a 24-well plate according to previous methods (Marikawa et al., 2009). Plasmids were transfected using Lipofectamine2000 (Invitrogen) according to the manufacturer’s instructions. Each experimental set contained a control well, four drug-treated wells (0.1, 1, 10, and 100 µM), and a positive control well (5 µM XAV939). Drugs were administered on the same day as plasmid transfection. The following plasmids were obtained commercially: TOPFLASH (Upstate), FOPFLASH (Upstate), and pRL-TK (Promega). The dual-luciferase assay was conducted using the Dual-Luciferase Reporter Assay System (Promega) with Gene Light 55 Luminometer (Microtech), according to the manufacturer’s instructions.
2.7. Statistical Analyses

For morphogenetic analyses, mean values of morphometric parameters were calculated for control EBs, which were then used to normalize drug-treated values of the same experimental set. For cell proliferation and dual-luciferase assays, control value was set to 100%, which was then used to normalize drug-treated values. At least three sets of experiments were performed for each drug using cell suspensions of different passage numbers as biological replicates. Statistical differences were measured using paired two-sample t-test. For morphogenetic analyses, t-test was performed for each experimental set. Drug impact was deemed significant if $p < 0.05$ in all three replicates. For cell proliferation and dual-luciferase assays, t-test was performed for a compiled data of the three replicates.
Chapter 3. Results

3.1. Morphogenetic impact of diabetes-related drugs on EB development

In this study, the axial elongation of P19C5 EBs (Lau & Marikawa, 2014) was used as an \textit{in vitro} model of early embryogenesis to examine the potential teratogenic impact of diabetes-related compounds in Selleck Chemicals’ Anti-diabetic Compound Library (Table 4). The library is composed of 31 compounds, which covers various mechanisms of actions and structures. Some drugs are currently marketed for treatment of diabetes and its complications, whereas others are marketed for other uses, still in clinical trials, no longer being pursued for use as an anti-diabetic, or used as an experimental tool to study diabetes. To examine the morphogenetic effect of these drugs, P19C5 EBs were cultured in four different concentrations of drug (0.1, 1, 10, and 100 µM) and in control (DMSO only), and the size and Elongation Distortion Index (EDI) of each EB was measured on Day 4 of culture. Details of morphogenetic effects are described for each compound below, and data is shown in Figure 4. Also, the overall summary of morphogenetic impact for all 31 compounds is presented as a schematic diagram in Figure 3.

3.1.1. \textit{Alpha-glucosidase inhibitors}

Acarbose and miglitol are alpha-glucosidase inhibitors (AGIs) that slow down the digestion of carbohydrates in the small intestine, and they are prescribed to treat type 2 diabetes. For acarbose, there was a consistent decrease in EB size at all concentration ranges tested (0.1, 1, 10, and 100 µM) in a dose-dependent manner, however, there was no consistent change in EDI (Fig. 4A). Acarbose-treated EBs were 20-25\% smaller than control EBs at 100 µM. Miglitol only showed a decrease in size at 100 µM, but like acarbose, there was no consistent
change in EDI (Fig. 4B).

3.1.2. Biguanides

Metformin HCl and Phenformin HCl are biguanides, which help lower blood glucose in type 2 diabetes patients by decreasing the amount of glucose produced by glycogenolysis and gluconeogenesis in the liver. However, their exact mechanisms of action are not fully known. Metformin HCl did not show any consistent change in size or EDI (Fig. 4C). Interestingly, phenformin HCl, showed a dose-dependent decrease in size (0.1, 1, 10, and 100 µM), and EBs were about 70% smaller than control EBs at 100 µM (Fig. 4D). Phenformin HCl-treated EBs also showed an increase in EDI at 10 and 100 µM. It must be noted that phenformin HCl was removed from the market because of a small number of cases of lactic acidosis, which was fatal in about half the cases. Metformin HCl, however, is still on the market.

3.1.3. Meglitinides

Mitiglinide calcium, nateglinide, and repaglinide are meglitinides, or nonsulfonylureas, which bind to ATP-sensitive K⁺ (K_{ATP}) channels to stimulate pancreatic beta-cells to increase insulin secretion to treat type 2 diabetes. Mitiglinide calcium showed a dose-dependent (0.1, 1, 10, and 100 µM) decrease in size, where EB size was about 30% less at 100 µM (Fig. 4E). Mitiglinide calcium-treated EBs, however, did not show any significant change in EDI at all concentrations. Like mitiglinide calcium, nateglinide-treated EBs also showed a dose-dependent (0.1, 1, 10, and 100 µM) decrease in size, where EB size was about 20-30% less than control EBs (Fig. 4F), but they showed no significant change in EDI at all concentrations. Like the other
glinides, repaglinide showed a dose-dependent (0.1, 1, 10, and 100 µM) decrease in size, and by 100 µM there was about a 40-50% decrease in EB size (Fig. 4G). But, unlike the other glinides, EDI was significantly decreased in repaglinide-treated EBs by 40-70% at 100 µM.

3.1.4. Sodium-dependent glucose transporter 2 inhibitors

Canagliflozin and dapagliflozin are sodium-dependent glucose transporter 2 (SGLT2) inhibitors. They work to treat type 2 diabetes by blocking glucose reabsorption in the proximal convoluted tubule in the kidney, which increases glucose excretion through urine. Canagliflozin-treated EBs decreased in size in a dose-dependent (0.1, 1, 10, and 100 µM) manner, and at 100 µM EB size was decreased by 83-84% (Fig. 4H). EDI was significantly increased only at 10 µM. Dapagliflozin-treated EBs were significantly reduced in size at 0.1, 1, 10 µM, but size decrease was not in a dose-dependent manner (Fig. 4I). At 100 µM EB size was significantly decreased by about 35-50%, and EDI was significantly decreased by about 65-75%.

3.1.5. Sulfonylureas

Gliclazide, glimepiride, glipizide, gliclazide, and glyburide are all sulfonylureas used to treat type 2 diabetes. They bind to ATP-sensitive K⁺ (K\textsubscript{\text{ATP}}) channels, specifically sulfonylurea receptors (SUR), on the cell membrane of pancreatic beta-cells, which stimulates the cells to release more insulin. Gliclazide treatment significantly decreased EB size only at 1 µM by approximately 12-14%, however, it had no effect on EDI for all concentrations (Fig. 4J). There was a dose-dependent (0.1, 1, 10, and 100 µM) decrease in EB size for glimepiride-treated EBs, and at 100 µM size was reduced by about 30-50% (Fig. 4K). Like gliclazide, glimepiride had no
effect on EDI. For glipizide-treated EBs, size was only significantly reduced at 10 µM (about 15-36% decrease) and 100 µM (about 22-33% decrease), and EDI was unaffected (Fig. 4L). Gliquidone treatment reduced EB size in a dose-dependent manner (0.1, 1, 10, and 100 µM), and at 100 µM size was reduced by about 37-48% (Fig. 4M). Unlike the other sulfonylureas in the compound library, gliquidone reduced EDI significantly at 100 µM to about 73-35% of that of control EBs. Glyburide, like gliclazide and glimepiride caused a dose-dependent (0.1, 1, 10, and 100 µM) decrease in size, where 100 µM glyburide resulted in about a 36-65% decrease, but there was no significant effect on EDI (Fig. 4N).

3.1.6. Dipeptidyl peptidase 4 inhibitors

Linagliptin, saxagliptin, sitagliptin phosphate monohydrate, and vildagliptin are dipeptidyl peptidase 4 (DPP4) inhibitors, and they work to treat type 2 diabetes by increasing hormone incretins, which inhibit glucagon release and lead to insulin secretion. P19C5 cells cultured in 100 µM linagliptin died by Day 1, which was unique to this gliptin, but at lower concentrations (0.1, 1, and 10 µM) there was a dose-dependent decrease in size, where EBs were 20-25% smaller than control at 10 µM (Fig. 4O). 10 µM linagliptin also decreased EDI by 25-50%. Saxagliptin-treated EBs and Sitagliptin phosphate monohydrate-treated EBs showed no significant change in size or EDI (Fig. 4P and 4Q). Vildagliptin showed a dose-dependent (0.1, 1, 10, and 100 µM) decrease in size, and at 100 µM there was about a 20-30% decrease in size with no consistent change in EDI (Fig. 4R).
3.1.7. Thiazolidinediones

Pioglitazone, pioglitazone HCl, rosiglitazone, rosiglitazone HCl, and rosiglitazone maleate are thiazolidinediones (TZDs) that activate peroxisome proliferator-activated receptor gamma isoform (PPARγ), which sensitizes adipocytes and myocytes to insulin. All the glitazones showed a dose-dependent (0.1, 1, 10, and 100 µM) decrease in size (Fig. 4S-4W). Pioglitazone and pioglitazone HCl both had significant decreases in size even at 0.1 µM, about 54-63% and about 59-68%, respectively, and they both also showed significant increase in EDI at 10 µM (Fig. 4S and 4T). For rosiglitazone, size is decreased in a dose-dependent manner (0.1, 1, 10, and 100 µM) and EDI is affected inconsistently (Fig. 4U). However, EB size was significantly reduced starting at 1 µM for rosiglitazone HCl and 10 µM for rosiglitazone maleate, and both drugs did not show any significant impact on EDI at all concentrations (Fig. 4V and 4W).

3.1.8. Others

Manidipine 2HCl is an antihypertensive agent that blocks L- and T-type Ca²⁺ channels to decrease blood pressure. This medication is used to treat hypertensive diabetics. P19C5 cells cultured in 100 µM manidipine 2HCl died by Day 1, but at lower concentrations (0.1, 1, and 10 µM) there was a dose-dependent decrease in size, where by 10 µM, EBs were 40-55% smaller than control EBs (Fig. 4X). Manidipine had no significant impact on EDI.

Resveratrol is a polyphenolic stilbene that has been shown to upregulate the Sirt1 gene as well as increase SIRT1 protein levels, which has been shown to sensitize cells to insulin, but the exact mechanism remains unknown. P19C5 cells cultured in 100 µM resveratrol died by Day 1, but at lower concentrations there was a dose-dependent (0.1, 1, and 100 µM) decrease in
size, and at 10 µM there was about a 77-86% decrease in EB size (Fig. 4Y). At all concentrations, resveratrol did not have a consistent impact on EDI.

Rucaparib is a poly ADP-ribose polymerase (PARP) inhibitor that inhibits PARP-1 and PARP-2. It is currently under clinical trials (Phase II and III) for cancer, specifically ovarian and pancreatic, however it was in a Phase II study to treat diabetic macular edema (MDE) but later withdrawn. Its mechanism of action as an anti-diabetic is not known. Rucaparib treatment resulted in death by Day 1 at 100 µM (Fig. 4Z), but at lower concentrations (0.1, 1, and 10 µM) there was a significant dose-dependent decrease in size, which resulted in about a 50-54% decrease at 10 µM. EDI was not consistently altered by rucaparib.

Streptozotocin is an alkylating agent used to inhibit DNA synthesis and alkylate glucose transporter 2 (GLUT2) in pancreatic beta-cells. It is a compound that is known to cause diabetes in animal models. Streptozotocin-treated EBs decreased in size in a dose-dependent manner (0.1, 1, 10, and 100 µM), and at 100 µM size was reduced by about 25-30% (Fig. 4AA). EDI was not significantly altered by streptozotocin.

GSK1292263 is a G-protein coupled receptor 119 (GPR119) agonist that was shown to decrease glucose levels by increasing glucagon-like peptide 1 (GLP-1), gastric inhibitory polypeptide (GIP), peptide YY (PYY) without affecting insulin levels. Currently, it appears to be in the preclinical trial phase for treating type 2 diabetes. There was a dose-dependent (1, 10, and 100 µM) decrease in EB size after GSK1292263 treatment, and resulted in about a 65-70% decrease in size at 100 µM. EDI was also significantly decreased at 100 µM by about 70-85% (Fig. 4BB).
LY2608204 is a glucokinase (GK) activator that completed a Phase I clinical trial for type 2 diabetes treatment and is now marketed for use in China. P19C5 cells cultured in 10 and 100 µM LY2608204 died by Day 1, but there was no significant change in size or EDI at 0.1 and 1 µM (Fig. 1 CC).

MK-8245 is a liver-targeted stearoyl-CoA desaturase (SCD) inhibitor that was in a preclinical trial for type 2 diabetes treatment. EB size was significantly decreased at 10 µM (about 30-35%) and 100 µM (about 20-40%), and EDI was significantly decreased at 1 µM (about 25-50%) and 10 µM (about 55-70%), but not at 100 µM (Fig. 1DD).

TAK-875 is a G-protein coupled receptor 40 (GPR40)/free fatty acid receptor 1 (FFAR1) agonist that increased glucose-dependent insulin secretion. It was developed for treatment of type 2 diabetes and reached Phase III clinical trials, however, due to liver safety concerns, the drug development was stopped. TAK-875 treatment resulted in death by Day 1 at 100 µM (Fig. 1EE), but at lower concentrations (0.1, 1, and 10 µM) there was a significant dose-dependent decrease in size, which resulted in about a 30-40% decrease by 10 µM. EDI was significantly increased at 10 µM by about 2-fold.

3.2. Temporal expression patterns of key regulatory genes are altered by anti-diabetic drugs

To understand how some selected diabetes-related drugs were impacting P19C5 EB morphogenesis at the molecular level, quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed on control and drug-treated Day 1, 2, 3, and 4 EBs. The P19C5 EBs were previously shown to display distinct temporal and spatial gene expression patterns of key developmental regulators, which correspond to those during primitive streak
formation, gastrulation, and body patterning (Lau & Marikawa, 2014). Because treatment with some drugs resulted in abnormal EB growth, I hypothesized that key developmental genes were misregulated by the drugs. In the present study, I focused on seven developmental genes, specifically, Pou5f1 (OCT4), Wnt3, Wnt3a, Brachyury (T), Tbx6, Fgf8, and Hoxc6, each of which represents distinct aspects of embryological events, as described below. Expression levels of these developmental regulators were normalized with the house-keeping gene Actb (encoding β-actin), as its expression is mostly consistent and unchanged during the four days of EB development (Marikawa et al., 2009; Lau & Marikawa, 2014).

Pou5f1 (peak expression on Day 0 in normal P19C5 EBs) encodes for a transcription factor Oct4, which plays a key role in the maintenance of an undifferentiated state and stem cell pluripotency. The Wnt genes (Wnt3 and Wnt3a) encode secreted signal proteins important for developmental processes, including cell fate determination and patterning in embryogenesis, and they are considered proto-oncogenes. Wnt3 (expressed the highest on Day 1 in normal EBs) is essential for initiating primitive streak formation (Tortelote et al., 2013), while Wnt3a (expressed the highest on Day 2 in normal EBs) is important for maintaining the primitive streak at the later stages (Wang, Sinha, & Wynshaw-Boris, 2012). Brachyury, which encodes for a nuclear transcription factor protein, is a downstream target of Wnt3 (expressed the highest on Day 1 in normal EBs), and its downstream targets are genes required for mesoderm and endoderm formation (Arnold et al., 2000). Tbx6 (expressed the highest on Day 2 in normal EBs), a downstream target of Wnt3a, encodes for a transcription factor important for the specification of paraxial mesoderm structures (Wittler et al., 2007). Studies suggest Fgf8 (expressed high on Day 1 in normal EBs), encodes a signaling molecule Fibroblast Growth Factor
8, and is involved in various aspects of embryogenesis, namely gastrulation, left-right axis determination, limb and brain development, and organogenesis (Sun, et al., 1999; Boettger, Wittler, & Kessel, 1999; Meyers & Martin, 1999; Lewandoski, Sun, & Martin, 2000; Storm, Rubenstein, & Martin, 2002; Abu-Issa et al., 2002). Finally, *Hoxc6* (steadily increases over 4 days of culture in normal EBs) is a member of the HOX gene family important for the anterior-posterior body patterning (Gilbert, 2014).

Upon qualitative examination of the drug effects on EB morphogenesis as described above (3.1), I chose to further examine four of the 31 anti-diabetic drugs at a specific concentration for gene expression analyses: dapagliflozin (100 µM), phenformin HCl (10 µM), manidipine 2HCl (10 µM), and resveratrol (10 µM). These drugs appeared to have the most dramatic effects on EB development, implicating that gene expression pattern may also be significantly altered by the drugs. EBs prepared from the same cell suspension were cultured with or without the drug, (the latter being the matching control in each set of experiment), collected everyday up to Day 4, and processed for qRT-PCR analysis for the above genes.

Dapagliflozin at 100 µM significantly decreased EB size and EDI, causing a smaller and rounder appearance. qRT-PCR analyses showed that dapagliflozin did not alter the expression pattern of *Pou5f1* in a consistent manner, as its level was robustly decreased by Day 1 and reached near the baseline by Day 2, similar to the control. In contrast, the *Wnt3* expression was dramatically reduced by dapagliflozin. The *Brachyury* peak level at Day 1 was also reduced by dapagliflozin. The *Wnt3a* peak at Day 2 appeared unchanged between control and dapagliflozin-treated EBs, although the *Tbx6*, peak was nearly absent in the latter. Dapagliflozin also delayed the up-regulation of *Fgf8* expression, shifting its peak to Day 2 from Day 1. The
expression levels of *Hoxc6* were consistently lower in the drug-treated EBs on Days 3 and 4. Thus, dapagliflozin impacted 5 out of 7 genes examined, including all 3 genes that normally peak on Day 1 (*Wnt3*, *Brachyury*, and *Fgf8*). This suggests that the initial step of primitive streak formation, which is controlled by Wnt/β-catenin signaling, may be compromised by dapagliflozin. This possibility is further tested in a later section (see 3.4; Fig. 5A).

Morphometric and statistical data analysis showed that phenformin HCl at 10 µM significantly lowered EB size while it increased EDI (Fig. 3), causing a smaller and skinnier appearance. qRT-PCR analysis showed that most of the key regulators examined, namely 6 out of 7 genes, were largely unaffected by phenformin HCl. The only gene that was consistently and distinctly altered by phenformin HCl was *Fgf8*, whose expression on Day 1 was significantly reduced (Fig. 5B).

Manidipine 2HCl at 10 µM caused consistent reduction in EB size without affecting EDI, and thus EBs appeared to be smaller but had a similar morphology to the control (Fig. 3). qRT-PCR analyses showed that manidipine 2HCl did not consistently alter the expression patterns of *Pou5f1* or *Wnt3*. However, the expression levels of *Brachyury* and *Fgf8* were consistently diminished by manidipine 2HCl at Day 1, whereas levels at Day 2 were higher than control at Day 2. Manidipine 2HCl significantly increased the peak level of *Wnt3a* on Day 2, although its downstream target *Tbx6* did not show consistent upregulation. *Hoxc6* was slightly but consistently lowered by manidipine 2HCl, even though its expression still steadily increased over the four days of culture period (Fig. 5C).

Resveratrol at 10 µM caused dramatic reduction in EB size (Fig. 3). Three out of the 7 genes were most dramatically affected by resveratrol: *Wnt3a*, *Tbx6*, and *Hoxc6*. The normal
Day 2 peaks for Wnt3a and Tbx6 were essentially abrogated by resveratrol, and Hoxc6 level remained low throughout the four-day culture period (Fig. 5D). Fgf8 was mildly but consistently affected by resveratrol, as its peak on Day 1 was reduced. Other genes, i.e., Pou5f1, Wnt3, Brachyury, were largely unaffected by resveratrol.

3.3. Impact of the four drugs on cell proliferation

All four of the chosen drugs examined for gene expression profiling reduced EB size, raising the possibility that they negatively impact cell proliferation. To test this possibility, P19C5 cells were cultured in a monolayer for 48 hours in either control (1% DMSO only) or drug-containing medium (0.1, 1, 10, and 100 µM with 1% DMSO), followed by measurement of cell numbers. In this cell proliferation assay, a monolayer culture, rather than a hanging drop culture, was employed because the former condition maintains the undifferentiated, homogeneous state of P19C5 cells. This should allow more specific assessment of drug impact on cell proliferation rather than its effect on cell differentiation or other various events that take place in EBs.

There was a dose-dependent decrease in cell numbers for dapagliflozin-treated P19C5 cells. Decrease was significant for 1, 10, and 100 µM, and there was a 50-60% decrease at 100 µM (Fig. 6A). Such dose-dependent decrease was also observed for Day 4 EB size (Fig. 3). Phenformin HCl also caused dose-dependent decrease in cell number: about 10-20% decrease at 0.1, 1, and 10 µM, and a drastic decrease by about 60% at 100 µM (Fig. 6B). This was also reflected in Day 4 EB size (Fig. 3). Manidipine 2HCl was cytotoxic at 100 µM as no cells survived 48 hours of monolayer culturing. This was consistent with the observation that cells failed to
form EBs in hanging drops containing 100 µM manidipine 2HCl (Fig. 3). At lower concentrations, manidipine 2HCl showed dose-dependent reduction in cell proliferation: about a 20-25% decrease at 0.1 and 1 µM and about a 45-50% decrease at 10 µM (Fig. 6C). The situation was similar for Day 4 EB size (Fig. 3). Resveratrol also exhibited cytotoxicity at 100 µM, while reducing cell proliferation in a dose-dependent manner at lower concentrations (Fig. 6D). These results show that, at least for the four drugs examined, overall impact on EB size was reflected by their negative effect on cell proliferation.

3.4. Inhibition of Wnt/β-catenin signaling by dapagliflozin and phenformin HCl

As described above (3.2), dapagliflozin downregulated Wnt3 expression. This may have resulted in a downregulation of Wnt/β-catenin, which in turn could have affected the expression of the other genes, namely Brachyury and Fgf8. To test whether Wnt/β-catenin signaling was indeed affected by dapagliflozin treatment, a TOPFLASH reporter assay was conducted. The TOPFLASH plasmid contains a luciferase reporter gene under the control of TCF/LEF response elements, and is widely used as an indicator of active Wnt/β-catenin signaling (Korinek et al., 1997). Monolayer P19C5 cells were transfected with either TOPFLASH, or negative control plasmid FOPFLASH containing mutated TCF/LEF binding sites, and at the same time treated with drug (0.1, 1, 10, and 100 µM in 1% DMSO), control (1% DMSO only), or a known pharmacological inhibitor of Wnt/β-catenin signaling XAV939 (5 µM in 1% DMSO) as a positive control. TOPFLASH signal significantly decreased at 100 µM dapagliflozin concentration (65-70% decrease; Fig. 7A). On the other hand, FOPFLASH signal was unaffected by dapagliflozin at all concentrations, ensuring that the impact of dapagliflozin on TOPFLASH signal was specific.
to the TCF/LEF-binding site, i.e., Wnt/β-catenin signaling, rather than on the basal promoter or on the luciferase enzymatic activity.

Phenformin HCl had similar results to dapagliflozin in the cell proliferation assay. In order to determine whether a decrease in cell proliferation was causing a decrease in Wnt/β-catenin signaling, I also tested the effects of phenformin HCl on the TOPFLASH reporter. Phenformin HCl, unlike dapagliflozin, had a dose-dependent decrease on Wnt/β-catenin signaling, and at 100 µM there was a 50-60% decrease in signaling (Fig. 7B). This observation raises the possibility that impact on cell proliferation may play a role in inhibition of Wnt/β-catenin signaling in P19C5 cells.
Chapter 4. Discussion

4.1. Morphogenetic impact of diabetes-related drugs

Chemical teratogens are difficult to identify because of their complex actions, which are greatly influenced by the timing and level of exposure to a developing embryo. For the present studies, the elongation morphogenesis of the P19C5 EBs was used as an *in vitro* model of gastrulation and body patterning to test the potential teratogenicity of various diabetes-related drugs. The overall results of morphogenetic effects for all 31 drugs in the Selleck Chemicals Anti-diabetic Compound Library is summarized in Figure 3. Overall, 27 of the 31 anti-diabetic compounds had some impact on EB size and 11 of 31 had some effect on EDI. The anti-diabetic drugs decreased EB size, particularly at the high concentration (100 µM), but they had variable effects on EDI. For example, Pioglitazone and TAK-875 increased EDI at 10 µM, but Linaglitpin and MK-8245 decreased EDI at 10 µM. Six of the 31 drugs resulted in EB death at 100 µM, and one of those, LY2608204, also killed EBs at 10 µM (Fig. 3). In general, drugs in the same class tended to exhibit similar morphogenetic effects, as in the thiazolidinedione and SGLT2 inhibitor classes. However, metformin HCl and phenformin HCl, while both are biguanides, displayed dissimilar effects on EBs, suggesting that their different chemical characteristics are responsible for distinct morphogenetic impact.

Further investigations are important to ensure that false-positive and false-negative results occur minimally. False-positive results could take potentially life-saving medications off the market unnecessarily. In contrast, false-negative results may allow potentially teratogenic compounds to fall into the hands of pregnant mothers. *In vivo* animal studies and human case studies may be useful to ensure that the drugs are truly teratogenic, but it must be emphasized
that such studies are time-consuming and expensive. Thus, initial drug screening processes can be conducted using *in vitro* assays, such as the P19C5 EB elongation morphogenesis model, which is relatively fast and easy to test the potential teratogenicity of many drugs.

### 4.2. Comparisons between human plasma and *in vitro* teratogenic concentrations

*In vitro* assays, such as the P19C5 morphogenesis model, are ideal for dose-response embryotoxicity studies because the exact concentration of a chemical exposed to the test system can be specified. This is unlike *in vivo* animal models, in which chemical concentrations around developing embryos are affected by variables such as metabolism by the mother, the ability to pass the placenta, and also the drug delivery route (e.g., intraperitoneal, oral, subcutaneous, or intravenous). The present study showed that different diabetes-related drugs exhibited morphogenetic impact on P19C5 EBs at specific concentrations. The concentrations of some diabetes drugs found in the circulating blood or plasma of diabetes patients have been estimated through human clinical studies. Such human plasma concentrations can be compared with those that impact EB morphogenesis, or “*in vitro* teratogenic concentrations,” which may provide information on how likely the drug could cause birth defects (Table 6). Drug plasma concentration was acquired at different time intervals, but it was typically acquired at specified time points from 0 to 24 hours post-dose. For many drugs, plasma concentrations are close to the lower concentrations tested on P19C5 EBs (0.1 to 1 µM), which did not impair morphogenesis in many cases. However, some of the diabetes-related drugs (e.g., MK-8245 and resveratrol) had morphogenetic impact even at the lower concentrations. Also, plasma
concentrations of some drugs (e.g., gliquidone and nateglinide) are relatively high and much closer to the higher concentrations tested on EBs (10 to 100 µM).

The plasma drug concentration, however, may not represent the true concentration that a developing embryo is exposed to because of factors such as the mother’s metabolism and placental transfer of the drugs. The time of exposure also affects the chemical concentration that the embryo is exposed to. For example, during the 3rd to 4th weeks of development, the embryo has not fully developed a placental barrier yet, so there may be increased exposure to some chemicals during this period compared to later stages of gestation.

While plasma concentration is often used to estimate exposure levels of drugs in the uterus, information on the true concentrations around the developing embryos is scarce. Amniocentesis, a method of removing a small amount of amniotic fluid, may be used to estimate drug concentrations surrounding the developing embryo, but this technique is moderately invasive and increases the possibility of miscarriage. Until a more effective and non-invasive method is developed to determine the exact drug concentration an embryo is exposed to, the tests we conduct need to be based on drug concentrations we think are reasonable to assess potential teratogenicity. Importantly, drug plasma concentrations can become much higher in patients with liver or kidney diseases that compromise drug metabolism and elimination. Thus, even when teratogenic effects are seen only at concentrations above typical plasma concentration ranges, such unusually high concentrations could arise in some patients.

In the present study, morphogenetic effects were investigated for individual drugs. However, in real-life situations, people often take more than one drug at the same time, and it is important to consider possible drug interactions. For example, one drug may inhibit
metabolizing enzymes in the liver or transporter channels in the kidneys, which would significantly increase the plasma concentration of other drugs taken. More than 50 percent of people with diabetes who take one type of medication will need to take another type within three years to maintain glucose homeostasis (Consumer Reports, 2012). Also, several marketed medications are already a combination of multiple drugs, such as metformin+glipizide (Metaglip™), metformin+glyburide (Glucovance™), pioglitazone+metformin (Actoplus Met™), and dapagliflozin+metformin HCl (Xigduo™). These combinatory medications may have a synergistic impact on EB/embryo development even at lower concentrations. An advantage of in vitro systems is that such multi-drug effects can be easily tested in a controlled environment, where variables are minimized and exact drug concentrations are specified.

4.3. Expression of key developmental genes are altered by diabetes-related drugs

To gain insight into the molecular mechanisms of drug effects on morphogenesis, I investigated temporal gene expression patterns of several developmental regulators, such as Pou5f1, Wnt3, Wnt3a, Tbx6, Brachyury, Fgf8, and Tbx6 (Fig. 5). My analyses revealed clear alterations in their expression profiles in response to drug treatment, which may be linked to the observed effects on EB growth and elongation morphogenesis. However, these are a limited set of genes involved in embryogenesis and morphogenesis, and thus a plethora of other genes critical for morphogenesis may also need to be analyzed in future studies. For example, Bmp4 is critical for early differentiation and establishing the dorsal-ventral axis (Schmidt, 1995), Shh is essential for early embryo patterning, such as the ventral neural tube, anterior posterior limb axis, and ventral somites (Ho & Scott, 2002; Marigo et al., 1996; Borycki,
Mendham, & Emerson, 1998), and Cdx2 is essential for axial patterning (Beck & Stringer, 2010). In addition to examining more genes via qRT-PCR, other assays can be conducted to analyze global gene expression patterns to better understand drug impact on EB morphogenesis. Such global analysis methods include microarrays, massively parallel signature sequencing (MPSS), and serial analysis of gene expression (SAGE). Although these methods are more costly than qRT-PCR, they are more time-effective and can yield more information. It is important to note that these methods, including qRT-PCR, can analyze gene expressions only at the mRNA level, and may not be suited for investigation of drugs that primarily affect protein synthesis, stability, and/or activity. Such protein-based information may be revealed through other methods, including western blotting, immunoprecipitation, and spectrophotometry.

Although my molecular investigations of drug effects were limited to qRT-PCR for several genes, they yielded significant insights into the potential mechanisms of four selected diabetes drugs that disrupted EB morphogenesis. Dapagliflozin (100 µM) decreased Wnt3, the key gene essential for primitive streak formation, which was reflected in reduction in Wnt/β-catenin signaling, as shown by the TOPFLASH reporter assay (Fig. 5A and 7A). In vivo studies have shown that knocking out Wnt3 results in the absence of a primitive streak, mesoderm, and node, and causes embryo death by E10.5 (Liu et al., 1999). This seems to coincide with the morphogenetic impact of dapagliflozin on EB development, where the EBs remained spherical, indicating that the drug-treated EBs failed to form a primitive streak, which is necessary for gastrulation and axial elongation. Tbx6 and Brachyury downregulation was also seen in dapagliflozin-treated EBs (Fig. 5A), which may have resulted from a downregulation in Wnt/β-catenin signaling, because these two genes are known transcriptional targets in mouse
embryos (Wittler et al., 2007; Arnold et al., 2000). How dapagliflozin interferes with Wnt/β-catenin signaling remains unclear; it may directly target Wnt3 or an upstream signal that is required for the activation of the Wnt3 gene. Because dapagliflozin is a known sodium-dependent glucose transporter 2 (SGLT2) inhibitor, it may be diminishing Wnt3 expression through inhibition of SGLT2. However, this possibility may be unlikely because the P19C5 EBs have little to no expression of the Slc5a2 gene, which encodes SGLT2, based on the preliminary microarray study in our lab. Furthermore, Slc5a2 knockout mice do not appear to exhibit embryological defects (Vallon et al., 2011). Nonetheless, another SGLT2 inhibitor tested in the present study, canagliflozin, also exhibited morphogenetic effects on EBs similar to dapagliflozin. Thus, the role of SGLT2 in EB morphogenesis needs to be further investigated in future studies, including verification of SGLT2 protein expression in EBs, gene expression analyses of canagliflozin-treated EBs, and testing of other SGLT2 inhibitors.

Interestingly, there may be a potential link between the anti-diabetic effects of dapagliflozin and its impact on Wnt signaling. TCFL2, an effector as well as a downstream target of Wnt signaling, is highly upregulated in the islets of patients with type 2 diabetes, and Wnt2b was also upregulated in both β-cells and α-cells of the pancreas (Lee et al., 2008). Thus, dapagliflozin may be alleviating hyperglycemia by decreasing Wnt signaling. My TOPFLASH reporter assay showed that dapagliflozin diminished Wnt signaling at 100 µM but not at 10 µM or lower concentrations (Fig. 7A). Therapeutic plasma concentrations of dapagliflozin in humans range from 0.299 µM to 1.35 µM, and thus dapagliflozin alone may not contribute a significant impact on Wnt signaling. However, it is possible that the Wnt-diminishing effect of dapagliflozin
may be potentiated in other cell types, such as pancreatic cells, or by other conditions or drugs in diabetes patients.

Phenformin HCl is a biguanide that was used to treat type 2 diabetes. However, the drug was discontinued for use because of a small number of cases of lactic acidosis, which was fatal half the time. Metformin HCl is another biguanide still used to treat diabetes today that does not seem to cause this problem. Biguanides help lower blood glucose by decreasing gluconeogenesis and glycogenolysis in the liver, but their exact mechanism of action is still not known. The TOPFLASH assay revealed that phenformin HCl exhibited negative impact on Wnt signaling in a dose-dependent manner (Fig. 7B). However, my qRT-PCR analyses showed that phenformin HCl (10 µM) did not decrease Tbx6 expression (Fig. 5B), a known downstream target of Wnt3a (Wittler et al., 2007), suggesting that Wnt/β-catenin signal was not significantly inhibited by the drug. This apparent discrepancy is likely due to the concentrations used for gene expression analysis. In the TOPFLASH assay, phenformin HCl at 10 µM inhibited Wnt signaling by about 30%, whereas dapagliflozin at 100 µM (which downregulated Tbx6) inhibited Wnt signaling by about 70% (Fig. 7). Insufficient inhibition of Wnt signaling by phenformin HCl at 10 µM is also consistent with its morphogenetic impact, i.e., EB size was diminished while EDI was increased (i.e., smaller and thinner looking). This suggests that the drug at this concentration did not interfere with the primitive streak formation. Interestingly, the other biguanide, metformin HCl, had no significant impact on EDI, which implicates that phenformin HCl may have another drug target in addition to the common targets of biguanides. An additional drug target may be what causes lactic acidosis with phenformin HCl and also be
linked to its unique morphogenetic impact, which may involve decreases in *Wnt3a* and *Fgf8* expression, as revealed by qRT-PCR analysis (Fig. 5B).

The *in vivo* human plasma concentration of manidipine 2HCl ranges from 2.11 nM to 7.03 nM, which is markedly less than the drug concentrations tested in the study (more than $10^3$-fold less). Nonetheless, it is possible that manidipine plasma concentration, or intrauterine concentration, may rise exceptionally high in some patients for the reasons described above (see 4.2). The qRT-PCR analysis of EBs treated with manidipine (10 µM) showed changes in gene expression profiles that appeared conflicting: the drug upregulated *Wnt3a*, but downregulated *Brachyury*, a downstream target of *Wnt3a* (Fig. 5C). It is possible that manidipine is targeting these two genes separately, or EBs are trying to compensate for the reduction in *Brachyury* by upregulating *Wnt3a* expression. Regardless, reduction in *Brachyury* was likely to have caused a delayed and decreased *Fgf8* expression, which was also observed in manidipine-treated EBs. Previous studies showed that *Brachyury* homozygous mutants have significantly reduced *Fgf8* expression at E8.0, indicating *Brachyury* is necessary for *Fgf8* expression (Evans *et al.*, 2012).

Manidipine is an inhibitor of L- and T-type calcium channels. But, whether inhibition of these channels is involved in the morphogenetic impact of manidipine is unclear. Such possibility may be unlikely because the P19C5 EBs show little to no expression of L-type (*Cacna1c, Cacna1d, Cacna1s*, and *Cacna1f*) and T-type (*Cacna1g, Cacna1h*, and *Cacna1l*) calcium channels, based on the preliminary microarray study in our lab.

Resveratrol has been shown to activate *Sirt1*, which encodes for a protein involved in histone modification, specifically NAD-dependent deacetylation, which is linked to the coordination of the cell cycle, metabolism, apoptosis, autophagy, and DNA-damage response.
Sirt1 is downregulated in diabetes model mice that exhibit insulin resistance, and upon administration of resveratrol, insulin sensitivity is improved. The action of resveratrol to treat diabetes appears to be mediated by activation of Sirt1, which suppresses Ptpb1, a negative regulator of the insulin signaling pathway at both mRNA and protein levels (Sun et al., 2007). Whether the morphogenetic impact of resveratrol on EBs is mediated by activation of Sirt1 is not clear, although the Sirt1 gene appears to be expressed robustly throughout EB development, according to the microarray data in our lab. Human plasma concentrations of resveratrol range from 0.19 µM to 4.24 µM, which is comparable to levels that impaired EB development, as EB size was significantly reduced at 1 µM. It should be noted that resveratrol is a dietary supplement with a dosage that is unregulated, so plasma concentrations may become much higher in people who take more than the recommended dose. My qRT-PCR analysis showed that resveratrol (10 µM) significantly downregulated Fgf8, Wnt3a, Tbx6, and Hoxc6 (Fig. 5D). As mentioned earlier, Wnt3a acts upstream of Tbx6 (Wittler et al., 2007), and thus reduction in Tbx6 may be secondary to downregulation of Wnt3a. Also, in an in vitro study using neural explants of chick embryos, Fgf8 administration induced Hoxc6 expression (Liu, Laufer, & Jessell, 2001), raising the possibility that reduction of Fgf8 in resveratrol-treated EBs is the cause of Hoxc6 downregulation. However, resveratrol at 10 µM caused substantial reduction in EB size (less than 20% of control at Day 4) and in cell numbers in a monolayer culture (about 20% of control after 48 hours; Fig. 6D). This indicates that at this concentration resveratrol markedly diminished cell proliferation or increased cell death, much more so than the other drugs and concentrations used for gene expression analyses. Because of
the extremely small EB size, it is possible that the mode of cell-cell interactions was significantly altered, which may have contributed to drastic changes in gene expression patterns. Further investigations are needed using lower concentrations of resveratrol that cause moderate morphological impact, which may shed light on more specific molecular targets of resveratrol.

4.4. Future of *in vitro* systems for teratogen studies

*In vitro* systems serve as a starting point for studies testing potential teratogenicity. The P19C5 EB system in particular can serve as a useful model for embryogenesis because of its unique ability to mimic aspects of primitive streak formation, gastrulation, and body patterning, which is evidenced by the distinct elongation morphogenesis as well as the temporal and spatial gene expression patterns seen in the EBs. This system, however, has its shortcomings like all other *in vitro* systems because it represents only limited regions and stages of embryogenesis. P19C5 EBs do not seem to exhibit features of cranial development, such as the heart, central nervous system (CNS), and craniofacial structures. This notion is based on the preliminary microarray data in our lab, which shows apparent lack of upregulation in master regulatory genes for the heart (e.g., *Nkx2.5* [Tanaka *et al.*, 1999]), CNS (e.g., *Sox1* [Pevny *et al.*, 1998]), and cranial (e.g., *Otx2* [Matsuo *et al.*, 1995]) formation during EB development.

Nonetheless, another study in our lab showed that EB morphogenesis is controlled by most of the major signaling pathways, including Wnt, Bmp, Nodal/Activin, Fgf, and retinoic acid, which are also involved in the formation of the heart, CNS, and craniofacial development (unpublished data). Thus, the P19C5 EB system, in spite of its limited representation of embryogenesis, may be able to detect a broader range of teratogens.
Another shortcoming of the P19C5 EB system is the potential interspecies discrepancy. P19C5 cells originated from mouse teratocarcinoma cells, not human cells, so the results from studying potential teratogenicity may not always be directly translatable to humans. Human and animal cells may potentially respond to chemical agents differently. For example, thalidomide, a known teratogen, had no effect on mouse and rat fetuses, even at extremely high doses (Fratta et al., 1965). Such differences can lead to false negative results that can have negative consequences, like in the tragic incidences of thalidomide babies. If an in vitro system is created using human embryonic stem cells (hESCs) to mimic many aspects of early embryogenesis, it would be a more relevant system to test the potential teratogenicity of chemicals because the hESCs would express genes similar to those seen in vivo in humans. It has been reported that under a specific culture condition, EBs of hESCs exhibit elongation morphogenesis in vitro (Lau & Marikawa, 2014). Whether these human EBs are behaving in a manner similar to P19C5 EBs, particularly with respect to gene expression patterns, is currently under investigation in our lab.

There is already a decrease in fertility, thus knowing whether the medications we take affects fertility and embryogenesis is important. The P19C5 EB system can serve as an initial screening process for the potential teratogenicity of chemicals. It must be emphasized that no single test or study can prove that a chemical is teratogenic, thus other studies must be conducted to confirm teratogenicity, and this is where in vivo animal models and epidemiological studies become useful. Ultimately, all those studies together should help people and doctors prescribe drugs safe for pregnant mothers. Our future preservation of fertility in women and maximization of healthy births rely on such studies.
<table>
<thead>
<tr>
<th></th>
<th>Physical Agents</th>
<th>Infectious Agents</th>
<th>Chemical Agents</th>
<th>Maternal Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Examples</td>
<td>Ionizing radiation, Hyperthermia</td>
<td>Toxoplasmosis, Rubella, Herpes simplex, Syphilis</td>
<td>Isotretinoin, ACE inhibitors, Thalidomide, Antibiotics</td>
<td>Diabetes, Malnutrition, Thyroid disorders</td>
</tr>
</tbody>
</table>
Table 2. The three approaches to identifying a teratogen

<table>
<thead>
<tr>
<th>Method</th>
<th>Examples</th>
<th>Pros</th>
<th>Cons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidemiological</td>
<td>Case control studies, Cohort studies</td>
<td>Accurate, Human Study</td>
<td>Too late, Many variables, Expensive</td>
</tr>
<tr>
<td>Studies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In vivo Animal</td>
<td>Mice, Rats, Rabbits, Non-human primates</td>
<td><em>In vivo</em>, Whole models</td>
<td>Expensive, Inter-species differences, Sacrifice animals</td>
</tr>
<tr>
<td>Studies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In vitro studies</td>
<td>Whole embryo culture tests, Organ culture teratogen assays, Cell culture systems</td>
<td><em>In vitro</em>, Cost-effective, Able to control variables, Simplified system</td>
<td>Not a whole organism, Can’t always extrapolate results</td>
</tr>
<tr>
<td>Class</td>
<td>Target Organ(s)</td>
<td>Mechanism of Action</td>
<td>Example(s)</td>
</tr>
<tr>
<td>-----------------------</td>
<td>-------------------</td>
<td>-------------------------------------------------------------------------------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>Sulfonylureas</td>
<td>Pancreas (Beta-cells)</td>
<td>Binds ATP-sensitive K(^+) channels (SUR) → Depolarization → Voltage-gated Ca(^{2+}) channels open → Exocytosis of insulin granules</td>
<td>Chlorpropamide (1(^{st}) gen.) Glipizide, gliclazide, and gliquidone (2(^{nd}) gen.) Glyburide (3(^{rd}) gen.) JB253 (4(^{th}) gen.)</td>
</tr>
<tr>
<td>Meglitinides (glinides)</td>
<td>Pancreas (Beta-cells)</td>
<td>Similar to sulfonyureas, except does not bind SUR</td>
<td>Repaglinide, mitiglinide, nateglinide</td>
</tr>
<tr>
<td>Dipeptidyl Peptidase-4 (DPP-4) Inhibitors (gliptins)</td>
<td>Pancreas (Beta-cells)</td>
<td>Inhibit DPP-4 → Increase hormone incretins, glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) → Inhibit glucagon → Increased insulin secretion</td>
<td>Sitagliptin, saxagliptin, linagliptin, alogliptin</td>
</tr>
<tr>
<td>Biguanides</td>
<td>Liver</td>
<td>Inhibit gluconeogenesis and glycogenolysis through unknown mechanism</td>
<td>Metformin, phenformin</td>
</tr>
<tr>
<td>Sodium-dependent Glucose Transporter 2 (SGLT2) Inhibitors</td>
<td>Kidney</td>
<td>Inhibit SGLT2 in proximal convoluted tubule → Decreased glucose reabsorption → Increased glucose excretion through urine</td>
<td>Canagliflozin, dapagliflozin</td>
</tr>
<tr>
<td>Thiazolidinediones (glitazones)</td>
<td>Liver</td>
<td>Activation of peroxisome proliferator-activated receptor gamma isoform (PPARY) → increased sensitization of adipocytes and myocytes to insulin</td>
<td>Pioglitazone, rosiglitazone</td>
</tr>
<tr>
<td>Bile Acid Sequestrants</td>
<td>GI System</td>
<td>Known action is LDL cholesterol-lowering; mechanism to lower blood glucose is unknown</td>
<td>Colesevelam</td>
</tr>
<tr>
<td>Alpha-glucosidase Inhibitors</td>
<td>GI System</td>
<td>Inhibit alpha-glucosidase in the small intestine, which inhibits carbohydrate breakdown</td>
<td>Miglitol, acarbose, voglibose</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-----------</td>
<td>----------------------------------------------------------------------------------</td>
<td>-----------------------------</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>Class</th>
<th>Target Organ</th>
<th>MOA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acarbose</td>
<td>Alpha-glucosidase Inhibitor</td>
<td>Adipocytes and Myocytes</td>
<td>Inhibits alpha-glucosidase</td>
</tr>
<tr>
<td>Miglitol</td>
<td>Alpha-glucosidase Inhibitor</td>
<td>Adipocytes and Myocytes</td>
<td>Inhibits alpha-glucosidase</td>
</tr>
<tr>
<td>Metformin HCl</td>
<td>Biguanide</td>
<td>Liver</td>
<td>Unknown</td>
</tr>
<tr>
<td>Phenformin HCl</td>
<td>Biguanide</td>
<td>Liver</td>
<td>Unknown</td>
</tr>
<tr>
<td>Repaglinide</td>
<td>Meglitinide</td>
<td>Pancreas</td>
<td>Bind $K_{\text{ATP}}$ Channels</td>
</tr>
<tr>
<td>Mitiglinide Calcium</td>
<td>Meglitinide</td>
<td>Pancreas</td>
<td>Bind $K_{\text{ATP}}$ Channels</td>
</tr>
<tr>
<td>Nateglinide</td>
<td>Meglitinide</td>
<td>Pancreas</td>
<td>Bind $K_{\text{ATP}}$ Channels</td>
</tr>
<tr>
<td>Canagliflozin</td>
<td>SGLT2 Inhibitor</td>
<td>Kidney</td>
<td>Inhibit SGLT2</td>
</tr>
<tr>
<td>Dapagliflozin</td>
<td>SGLT2 Inhibitor</td>
<td>Kidney</td>
<td>Inhibit SGLT2</td>
</tr>
<tr>
<td>Glimepiride</td>
<td>Sulfonylurea</td>
<td>Pancreas</td>
<td>Bind $K_{\text{ATP}}$ Channels (SUR)</td>
</tr>
<tr>
<td>Glipizide</td>
<td>Sulfonylurea</td>
<td>Pancreas</td>
<td>Bind $K_{\text{ATP}}$ Channels (SUR)</td>
</tr>
<tr>
<td>Glyburide</td>
<td>Sulfonylurea</td>
<td>Pancreas</td>
<td>Bind $K_{\text{ATP}}$ Channels (SUR)</td>
</tr>
<tr>
<td>Gliclazide</td>
<td>Sulfonylurea</td>
<td>Pancreas</td>
<td>Bind $K_{\text{ATP}}$ Channels (SUR)</td>
</tr>
<tr>
<td>Gliquidone</td>
<td>Sulfonylurea</td>
<td>Pancreas</td>
<td>Bind $K_{\text{ATP}}$ Channels (SUR)</td>
</tr>
<tr>
<td>Linagliptin</td>
<td>DPP-4 Inhibitor</td>
<td>Pancreas</td>
<td>Inhibit DPP-4</td>
</tr>
<tr>
<td>Saxagliptin</td>
<td>DPP-4 Inhibitor</td>
<td>Pancreas</td>
<td>Inhibit DPP-4</td>
</tr>
<tr>
<td>Sitagliptin</td>
<td>DPP-4 Inhibitor</td>
<td>Pancreas</td>
<td>Inhibit DPP-4</td>
</tr>
<tr>
<td>Vildagliptin</td>
<td>DPP-4 Inhibitor</td>
<td>Pancreas</td>
<td>Inhibit DPP-4</td>
</tr>
<tr>
<td>Pioglitazone</td>
<td>Thiazolidinedione</td>
<td>Liver</td>
<td>Activate PPARγ</td>
</tr>
<tr>
<td>Pioglitazone HCl</td>
<td>Thiazolidinedione</td>
<td>Liver</td>
<td>Activate PPARγ</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>Thiazolidinedione</td>
<td>Liver</td>
<td>Activate PPARγ</td>
</tr>
<tr>
<td>Rosiglitazone HCl</td>
<td>Thiazolidinedione</td>
<td>Liver</td>
<td>Activate PPARγ</td>
</tr>
<tr>
<td>Rosiglitazone maleate</td>
<td>Thiazolidinedione</td>
<td>Liver</td>
<td>Activate PPARγ</td>
</tr>
<tr>
<td>Compound</td>
<td>Category</td>
<td>Other Function</td>
<td>Effect</td>
</tr>
<tr>
<td>-----------------------</td>
<td>------------------------</td>
<td>-----------------------------</td>
<td>------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Manidipine 2HCl</td>
<td>Antihypertensive Agent</td>
<td>Other</td>
<td>Inhibit L- and T-type Ca(^{2+}) Channels</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>Polyphenolic Stilbene</td>
<td>Other</td>
<td>Activates <em>Sirt1</em> and SIRT1</td>
</tr>
<tr>
<td>Rucaparib</td>
<td>PARP Inhibitor</td>
<td>Other</td>
<td>Inhibits PARP</td>
</tr>
<tr>
<td>Streptozotocin</td>
<td>Alkylating Agent</td>
<td>Other</td>
<td>Alkylates GLUT2</td>
</tr>
<tr>
<td>GSK1292263</td>
<td>GPR119 Agonist</td>
<td>Other</td>
<td>Activates GPR119</td>
</tr>
<tr>
<td>LY2608204</td>
<td>Glucokinase Activator</td>
<td>Other</td>
<td>Activates GPR40/FFAR1</td>
</tr>
<tr>
<td>MK-8245</td>
<td>SCD Inhibitor</td>
<td>Other</td>
<td>Inhibits SCD</td>
</tr>
</tbody>
</table>

*Sitagliptin Phosphate Monohydrate*
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actb</td>
<td>5'-GAGAGGGAAATCGTGCGTGACATC</td>
<td>5'-CAGCTCAGTAACAGTCCGCCTAGA</td>
</tr>
<tr>
<td>Brachyury</td>
<td>5'-CCTCGGATTCACATCGTAGAGTT</td>
<td>5'-AGTAGGTTGGGCTGGCGTTATGACT</td>
</tr>
<tr>
<td>Dll1</td>
<td>5'-TGCCCACACGTCTATCTTGGATTA</td>
<td>5'-GTCACATAGACCCGAAGTGGCTTT</td>
</tr>
<tr>
<td>Fgf8</td>
<td>5'-GTTCGACTTGCGTTCTCTGCTT</td>
<td>5'-AGTCCTTGCGTTCTGCTCT</td>
</tr>
<tr>
<td>Hoxc6</td>
<td>5'-TCGCCACAGGAGAATGCGTGT</td>
<td>5'-CGAGTATAGGTAGCGGTTGAGTGA</td>
</tr>
<tr>
<td>Meox1</td>
<td>5'-AAAAATCAGACTTCCCCAGCGACAG</td>
<td>5'-TTCACACGTTTCCACTTCATCT</td>
</tr>
<tr>
<td>Mesp2</td>
<td>5'-GTCGCTTGGAAGTGGCCTTTATCTG</td>
<td>5'-GATACCTAGAAGCGGGGTGCTT</td>
</tr>
<tr>
<td>Notch1</td>
<td>5'-GTCTGCAGGCTCCAGTTGCTGA</td>
<td>5'-TCAGTTGGATTTGATGATGCT</td>
</tr>
<tr>
<td>Pou5f1</td>
<td>5'-AGGCAGGAGCAAGATGAAAGCA</td>
<td>5'-GAGGCTTGCCTGGGACTCAGAGA</td>
</tr>
<tr>
<td>Snai1</td>
<td>5'-CCGTCCAGCTGGAACCATGCGTCA</td>
<td>5'-TGGGAGACACATTTGGGAGAGCT</td>
</tr>
<tr>
<td>Tbx6</td>
<td>5'-GGCCTCCTCTCCACCCTTTAGTTC</td>
<td>5'-CAGTAGAACAAGGGCCCAGGAG</td>
</tr>
<tr>
<td>Wnt3</td>
<td>5'-CAGATGGCCCGTCACTATGAAACA</td>
<td>5'-AGCAAGCAGATGGAGACAGCAAT</td>
</tr>
<tr>
<td>Wnt3a</td>
<td>5'-GCCACAAGAGCCTCTGATAGGTA</td>
<td>5'-CCAGGCAAGACAGTACAGTCAGG</td>
</tr>
</tbody>
</table>

Table 5. Primer sequences for quantitative reverse transcription polymerase chain reaction
### Table 6. Human plasma and *in vitro* teratogenic concentrations

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose Used in Study</th>
<th>Time of Plasma Concentration Acquisition Postdose</th>
<th>Human Plasma Concentration (µM)</th>
<th>In Vitro Concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acarbose</td>
<td>NF</td>
<td>NF</td>
<td>NF</td>
<td>0.1 (Size)</td>
</tr>
<tr>
<td>Miglitol</td>
<td>50 mg</td>
<td>0 – 24 hrs</td>
<td>6.83 – 7.07</td>
<td>100 (Size)</td>
</tr>
<tr>
<td>Metformin HCl</td>
<td>500, 850 mg</td>
<td>1.79 – 4.01 hrs**</td>
<td>6.22 – 14.79</td>
<td>None</td>
</tr>
<tr>
<td>Phenformin HCl</td>
<td>NF</td>
<td>NF</td>
<td>NF</td>
<td>0.1 (Size), 10 (EDI)</td>
</tr>
<tr>
<td>Repaglinide</td>
<td>0.5, 1, 2, 4 mg</td>
<td>0 – 5 hrs</td>
<td>0.021 – 0.15</td>
<td>1 (Size), 100 (EDI)</td>
</tr>
<tr>
<td>Mitiglinide Calcium</td>
<td>5, 10, 20 mg</td>
<td>0.27 – 0.34 hr**</td>
<td>1.04 – 2.57</td>
<td>1 (Size)</td>
</tr>
<tr>
<td>Nateglinide</td>
<td>120 mg</td>
<td>0 – 12 hrs</td>
<td>17.93</td>
<td>1 (Size)</td>
</tr>
<tr>
<td>Canagliflozin</td>
<td>50, 100, 300 mg</td>
<td>0 – 24 hrs</td>
<td>0.96 – 10.52</td>
<td>1 (Size), 10 (EDI)</td>
</tr>
<tr>
<td>Dapagliflozin</td>
<td>5, 10 mg</td>
<td>0 – 96 hrs</td>
<td>0.30 – 1.35</td>
<td>1 (Size), 100 (EDI)</td>
</tr>
<tr>
<td>Glimepiride</td>
<td>1, 2, 4, 8 mg</td>
<td>1.6 – 3.2 hrs**</td>
<td>0.21 – 1.12</td>
<td>1 (Size)</td>
</tr>
<tr>
<td>Glipizide</td>
<td>5, 10 mg</td>
<td>0 – 24 hrs</td>
<td>1.48 – 1.74</td>
<td>10 (Size)</td>
</tr>
<tr>
<td>Glyburide</td>
<td>10 mg</td>
<td>0 – 24 hrs</td>
<td>0.55 – 0.60</td>
<td>0.1 (Size)</td>
</tr>
<tr>
<td>Gliclazide</td>
<td>160 mg</td>
<td>0 – 72 hrs</td>
<td>43.60 – 46.38</td>
<td>1 (Size)</td>
</tr>
<tr>
<td>Gliquidone</td>
<td>30 mg</td>
<td>0 – 16 hrs</td>
<td>0.23 – 4.06</td>
<td>1 (Size), 100 (EDI)</td>
</tr>
<tr>
<td>Linagliptin</td>
<td>5 mg</td>
<td>1.5 hrs**</td>
<td>8.9 nM</td>
<td>1 (Size), 10 (EDI)</td>
</tr>
<tr>
<td>Saxagliptin</td>
<td>2.5, 5 mg</td>
<td>2 – 4 hrs**</td>
<td>0.072 – 0.14</td>
<td>None</td>
</tr>
<tr>
<td>Sitagliptin*</td>
<td>100 mg</td>
<td>1 – 4 hrs**</td>
<td>0.95</td>
<td>None</td>
</tr>
<tr>
<td>Vildagliptin</td>
<td>100 mg</td>
<td>0.25 – 168 hrs</td>
<td>1.96</td>
<td>1 (Size)</td>
</tr>
<tr>
<td>Pioglitazone</td>
<td>45 mg</td>
<td>0 – 72 hrs</td>
<td>3.15</td>
<td>0.1 (Size), 10 (EDI)</td>
</tr>
<tr>
<td>Pioglitazone HCl</td>
<td>45 mg</td>
<td>2 – 3 hrs**</td>
<td>3.52</td>
<td>0.1 (Size), 10 (EDI)</td>
</tr>
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<td>Rosiglitazone</td>
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*Sitagliptin Phosphate Monohydrate; **Time of peak plasma concentration, T<sub>max</sub>; ***Chemosensitizing dose; Not Found, NF; Not Applicable, N/A
Figure 1. Chart showing the causes of birth defects. Approximately 20% of all birth defects are caused by genetic factors, including chromosome abnormalities and gene mutations. Approximately another 10% is caused by environmental factors, known as teratogens. A combination of genetic and environmental factors account for approximately another 20%, but about half of all birth defects have an unknown cause (modified from [Cleveland Clinic, 2012]).
Figure 2. Time-course of control P19C5 EB elongation morphogenesis. EBs form into spherical aggregates on Days 1 and 2, elongate slightly by Day 3, and are distinctly elongated by Day 4. The EBs naturally have a darker tint that marks the cranial end.
<table>
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* Sitagliptin Phosphate Monohydrate

**Figure 3.** Overall summary of morphogenetic impact for all 31 anti-diabetic compounds. A simple schematic showing whether the drug caused a decrease (red) or increase (green) in EB size and EDI, death (black), or did not affect (white) EBs.
Acarbose

**Figure 4. Morphogenetic impact data for each of 31 anti-diabetic drugs.** Representative photos are shown for each drug-treated set. Relative size and EDI are shown in the graphs. Each set of bars (blue, red, and green) represents one experimental set. Scale bar, 500 µm. *denotes significance for all three sets. Each drug was tested at least three times (Student’s t-test p<0.05).
Figure 4. (Continued) Morphogenetic impact data for each of 31 anti-diabetic drugs.
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Figure 4. (Continued) Morphogenetic impact data for each of 31 anti-diabetic drugs.

K Glimepiride
Gliplizide

Figure 4. (Continued) Morphogenetic impact data for each of 31 anti-diabetic drugs.
Figure 4. (Continued) Morphogenetic impact data for each of 31 anti-diabetic drugs.
Glyburide

Figure 4. (Continued) Morphogenetic impact data for each of 31 anti-diabetic drugs.
Linagliptin

Figure 4. (Continued) Morphogenetic impact data for each of 31 anti-diabetic drugs.
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S Pioglitazone
Figure 4. (Continued) Morphogenetic impact data for each of 31 anti-diabetic drugs.
Figure 4. (Continued) Morphogenetic impact data for each of 31 anti-diabetic drugs.
Rosiglitazone HCl

Figure 4. (Continued) Morphogenetic impact data for each of 31 anti-diabetic drugs.
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Figure 4. (Continued) Morphogenetic impact data for each of 31 anti-diabetic drugs.
Z Rucaparib

Figure 4. (Continued) Morphogenetic impact data for each of 31 anti-diabetic drugs.
Figure 4. (Continued) Morphogenetic impact data for each of 31 anti-diabetic drugs.
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Figure 4. (Continued) Morphogenetic impact data for each of 31 anti-diabetic drugs.
Figure 4. (Continued) Morphogenetic impact data for each of 31 anti-diabetic drugs.
A Dapagliflozin

Figure 5. (legend on the next page)
Figure 5. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) of developmental regulator genes. Pou5f1 (OCT4), Wnt3, Wnt3a, Brachyury (T), Tbx6, Fgf8, and Hoxc6 expression were investigated using qRT-PCR. A Results for dapagliflozin-treated EBs. B Mixl1 was also investigated for phenformin HCl-treated EBs. C Foxc2, Meox1, and Notch1 were also investigated for manidipine 2HCl-treated EBs. D Hoxa1 and Hoxb9 were also investigated for resveratrol-treated EBs. Horizontal axes represents culture time and vertical axes represents relative expression levels. Blue lines represent control EBs, and red lines represent drug-treated EBs. Data was normalized with the house-keeping gene, Actb. Experiments were performed at least three times and representative graphs are shown. * denotes significant differences between drug-treated and control values (Student’s t-test $p<0.05$).
Figure 5. (Continued) qRT-PCR of developmental regulator genes.
Figure 5. (Continued) qRT-PCR of developmental regulator genes.
D Resveratrol

![Graphs of developmental regulator genes](image)

Figure 5. (Continued) qRT-PCR of developmental regulator genes.
Figure 6. Cell proliferation is perturbed by anti-diabetic drugs. Cell proliferation assays were conducted over a 48-hour period for A dapagliflozin-treated EBs, B phenformin HCl-treated EBs, C manidipine 2HCl-treated EBs, and D resveratrol-treated EBs. Cells were cultured in control (1% DMSO) or drug-treatment (0.1, 1, 10, and 100 µM) conditions. Cells were counted using a hemocytometer. Assays were performed three times for each drug. Vertical axes represents cell number relative to control. *denotes significant differences between control and drug-treated EBs for all three sets (Student’s t-test $p<0.05$).
Figure 6. (Continued) Cell proliferation is perturbed by anti-diabetic drugs.
Figure 7. Wnt/β-catenin-signaling is affected by dapagliflozin and phenformin HCl. Dual-luciferase assays were conducted using control (1% DMSO), drug-treatment (0.1, 1, 10, and 100 µM), and 5 µM XAV939 (positive control) for A dapagliflozin and B phenformin HCl. TOPFLASH reporter was used to quantify Wnt/β-catenin signaling. FOPFLASH reporter was used to ensure drug-treatment was not affecting the TOPFLASH promoter. All tests were conducted three times each. Vertical axes represents relative luciferase activity. *denotes significant differences between control and drug-treated EBs for all three sets (Student’s t-test p<0.05).
Figure 7. (Continued) Wnt/β-catenin-signaling is affected by dapagliflozin and phenformin HCl.


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