INVESTIGATING THE EFFECTS OF BISPHENOL A ON THE HUMAN PLACENTA

A thesis submitted for the degree of Doctor of Philosophy

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September 2018

Acknowledgements

First of all, I would like to thank my principal supervisor, Dr. Emmanouil Karteris, for the inspiration to pursue this thesis and all the insights and help I have received along the way. Thank you for always having a solution to every problem and an open door to your office. To all the people who have helped me in the lab: Dr. Dimple Chudasama, Phoebe Maund, Jeyarooban Jeyaneethi and Perikles Katopodis. Your help is much appreciated. Dr Elisabete Silva, thank you for the insights and guidance as well as help with any troubleshooting issues.

As to my family, thank you for always supporting me from a distance, in whatever I do. And finally, to my husband, João. I appreciate all the moral support, and for sticking with me through it all!

Abstract

Investigating the Effects of Bisphenol A on the Human Placenta

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Introduction: Endocrine disrupting chemicals are environmental toxicants that humans are exposed to. Bisphenol A is classified as an endocrine disrupting chemical with xenoestrogenic capacity. The placenta is one of the least researched human organs, although it is crucial for the development of the embryo and fetus, and abnormal placental physiology can cause gestational complications that can lead to pregnancy determination. As such, we have elucidated the effects of bisphenol A in physiologically relevant doses on placental cell lines as well as human placental cells **Methods:** qPCR, Western blot, immunofluorescence, image stream, ELISA, microarray, 3D cell culture. **Results:** In placental cell lines BeWo and JEG-3, estrogen receptor α was the predominant receptor ($p>0.001$) in both non-syncytialised BeWo cells and in JEG-3 cells. 3 nM BPA treatment significantly increased cell proliferation in BeWo cells compared to controls ($p<0.05$), and this increase in cell proliferation was most likely due upregulation of estrogen receptor α ($p<0.001$) via a pathway involving p-p38 or p-AKT. Using microarray, pathways involving development of metabolic diseases such as type II diabetes, obesity and hypertension were significantly enriched in both the BeWo cell line and human placental cells after bisphenol A treatment. Finally, 3D models for placental culture were tested, showing that the 3D environment produces more physiologically relevant models of the human placenta, and methods prolonging the life of placental explants to up to 16 days were successfully developed. **Conclusion:** Bisphenol A in physiologically relevant doses changes the physiology of the human placenta via an upregulation of estrogen receptor α, causing an increase of cell proliferation and upregulating pathways that may result in the development of metabolic diseases, possibly exerting effects as early as fetal development. 3D models of human placenta should be used as a more physiologically relevant model of the human placenta when investigating these issues further.

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Chapter 1

1.1 Introduction

Endocrine disrupting chemicals (EDCs) are chemicals found in the environment, including chemicals in manufacturing and packaging materials, with the potential of disrupting the endocrine system of humans and wildlife. EDCs are widespread in the environment, and can accumulate all throughout the food chain, often due to long half-lives that are commonly found in these lipophilic chemicals and an inability of these compounds to be metabolized by the body (Montes-Grajales, Fennix-Agudelo, & Miranda-Castro, 2017). However, even if EDCs are metabolised, the resulting metabolites can be even more toxic than the original chemical itself (Diamanti-Kandarakis et al., 2009) for endocrine target organs. The endocrine system is vital for a multitude of necessary processes of the body, such as sexual differentiation in utero, sexual reproduction and growth, and every day processes such as metabolism, energy balance, homeostasis, and functioning of the cardiovascular system (Witorsch, 2002).

Long-term effects on development due to EDCs are associated with alterations in the epigenome, a phenomenon that confirms theories that adult onset diseases can be pre-defined through fetal exposure during development (Barker, 1997; Skinner, Manikkam, & Guerrero-Bosagna, 2011). The constant exposure of humans to these chemicals can cause metabolic dysfunction, disorders of the reproductive system, endocrine-related cancers and neurodevelopmental diseases (Koch & Diamanti-Kandarakis, 2015). The group of chemicals categorized as EDCs includes molecules that are used as industrial solvents or lubricants and also their by-products, pesticides and fungicides, chemicals found in plastics such as bisphenol A (BPA), phthalates used as plasticizers and agents used for pharmaceutical purposes. Natural chemicals found in food such as phytoestrogens can also be classified as endocrine disruptors (Diamanti-Kandarakis et al., 2009). According to the WHO-State of the science of endocrine disrupting chemicals-2012, around 800 chemicals are now known to have the potential of interfering with hormone receptors, the synthesis or conversion of hormones, although most of these chemicals have not been sufficiently investigated (Bergman, Heindel, Jobling, Kidd, & Zoeller, 2012). Although most studies have

focused on steroidal pathways, there is growing evidence that other hormonal and metabolic systems can be affected, leading to metabolic syndrome (Casals-Casas & Desvergne, 2011). In fact, EDCs can act through a multitude of pathways and binding partners, including nuclear and membrane-bound steroid hormone receptors, and can act by affecting enzymatic pathways necessary for steroid synthesis and metabolism, among others (Diamanti-Kandarakis et al., 2009).

Unlike hormones and other peptides, EDCs do not always follow a standard dose-effect curve. Therefore, there may not exist a minimal dose at which EDCs are ineffective (Crews, Willingham, & Skipper, 2000). Due to their probable accumulative nature, and for reasons yet unknown, EDCs exhibit a latency in their effect, showing symptoms years after initial exposure in humans. Furthermore, EDCs are rarely found alone in nature, and compounds combining EDCs may work additively or cumulatively, leading to a multitude of possible outcomes and effects, possibly having an additive effect if they work at the same target (Crews et al., 2000). It is therefore important to understand how EDCs interact in order to correctly postulate their effects on the human body. The main route of human exposure to EDCs is thought to be through dietary exposure, as EDCs such as organochlorine pesticides can be found in animal products used as foods (Acerini & Hughes, 2006). Other routes of exposure include air, drinking water, and household and cosmetic products that come into contact with skin. Newborns and fetuses can also be exposed via the lactational or transplacental route *in utero* respectively (Acerini & Hughes, 2006), and, later on, may also come in to contact with EDCs through ingestion of formula and via plastic baby bottles.

Understanding the effects of environmental chemicals during gestation is crucial, as normal fetal development paves the way for healthy development and growth in the child and adult. Although EDCs may have an effect in adulthood, the marked difference between exposure during development and exposure during adulthood is the irreversibility of an EDCs effect during a critical stage of development (Bigsby et al., 1999), especially *in utero*. EDCs have shown to have a multitude of effects on the fetus and infant, as hormonal regulation is the cornerstone of *in utero* sexual differentiation and development. The central nervous system (CNS) and immune system are also affected by sex steroids (Bigsby et al., 1999), and as most EDCs mimic estrogens or androgens in some way, these systems can be affected also.

Reproductive abnormalities are most commonly associated with xenoestrogens. These chemicals include BPA, polychlorinated biphenyls (PCBs) and phthalates which act as antiandrogens and therefore can be considered in this group. As these compounds all show steroid-like properties and can bind to corresponding receptors, any point in reproductive development along the axis of the hypothalamus to the gonad can be affected (Robins, Marsit, Padbury, & Sharma, 2011). Furthermore, BPA accumulates in the amniotic fluid, at levels 5 times higher than in maternal sera (Ikezuki, Tsutsumi, Takai, Kamei, & Taketani, 2002), probably due to the active transport of BPA across the placental membrane (Robins et al., 2011) a phenomenon that further highlights the harmful effects of EDCs during pregnancy.

1.2 Literature Review

1.2.1. Placental Physiology

1.2.1.1. Overview

The placenta is the most crucial organ when it comes to maintaining pregnancy and the development of the fetus and is the first organ that develops after the blastocyst attaches to the wall of the uterus (Aplin, Haigh, Vicovac, Church, & Jones, 1998; Davies et al., 2016). Not only do nutrients pass from the mother to the fetus and toxins get filtered through the placenta, but the placenta itself is an endocrine organ, highly sensitive to external signals and vital in regulation of the pregnancy, the feto-placental unit and maternal physiology, to adapt to the dynamic demands of pregnancy (Costa, 2016; Davies et al., 2016). For this reason, changes in the development of the placenta can cause adverse effects on the pregnancy and the way the fetus copes with the intrauterine environment (Gude, Roberts, Kalionis, & King, 2004). The main functions of the placenta include providing the fetus with water, oxygen, nourishment in the form of carbohydrates, lipids, amino acids vitamins and minerals, and removing waste products such as carbon dioxide. It also metabolises compounds, releasing metabolites into the maternal and/or fetal blood stream (Carter, Moores, & Battaglia, 1991; Costa, 2016). The trophoblast is the main unit of placental transport, with a maternal “brush-border” side and a basal side, which faces the fetal circulation

(Yudilevich & Barros, 1990). It also acts as a transient endocrine organ, releasing hormones that affect the pregnancy, growth, parturition and metabolism among other functions (Gude et al., 2004).

1.2.1.2 Early placental development

The placenta begins to form in a process called placentation, in which the outer part of the cell mass, or trophoblastic cells, differentiate and divide into the cytotrophoblast, which forms the connection between the embryo and the placenta, and the syncytiotrophoblast, which is a mass of differentiated cells that form large, multinucleated trophoblastic cells through a process called syncytialisation. The trophoblasts can take one of two pathways; the extravillous or the intravillous pathway, the syncytiotrophoblast being intervillous while the cytotrophoblast remains extravillous.

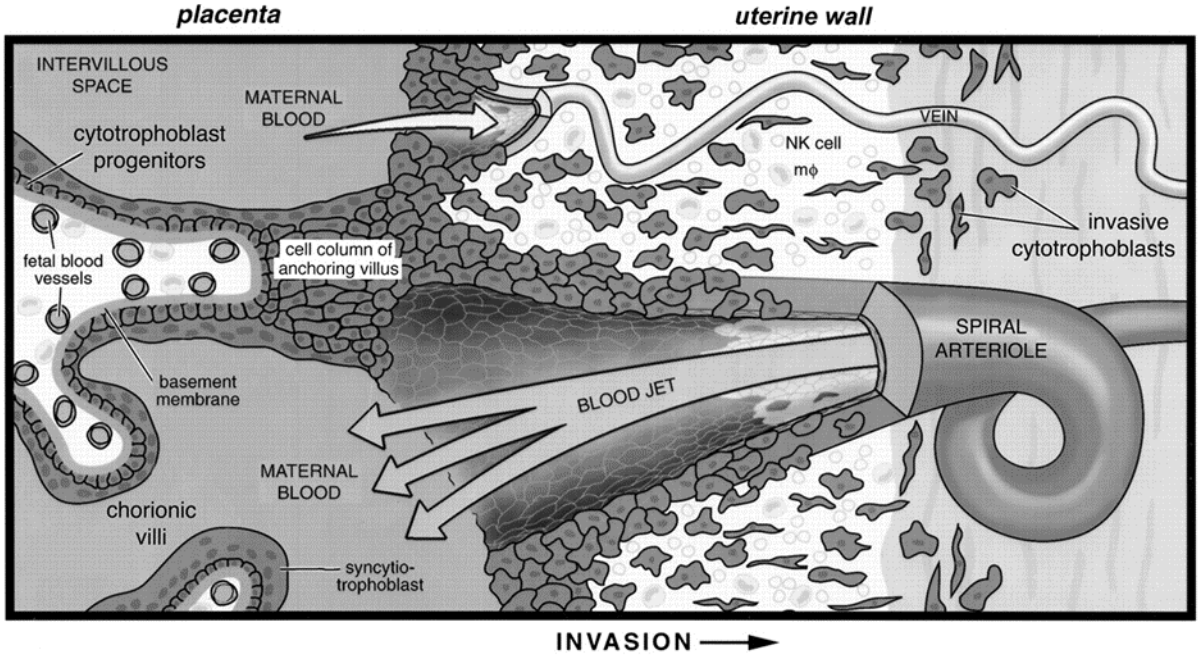

Figure 1.1 Different cells of the placenta and the structural subunits and mechanisms of invasion (Red-Horse et al., 2005). Invasive cytotrophoblasts invade into the uterine wall and spiral arterioles of the uterus, restructuring the vessels and creating a connection between maternal and fetal circulation. Other extravillous cytotropholasts invade the decidua and play a role in imunity, among other roles. The syncytiotrophoblast lines the chorionic villi as the main cell/structure regulating exchange between mother and fetus.

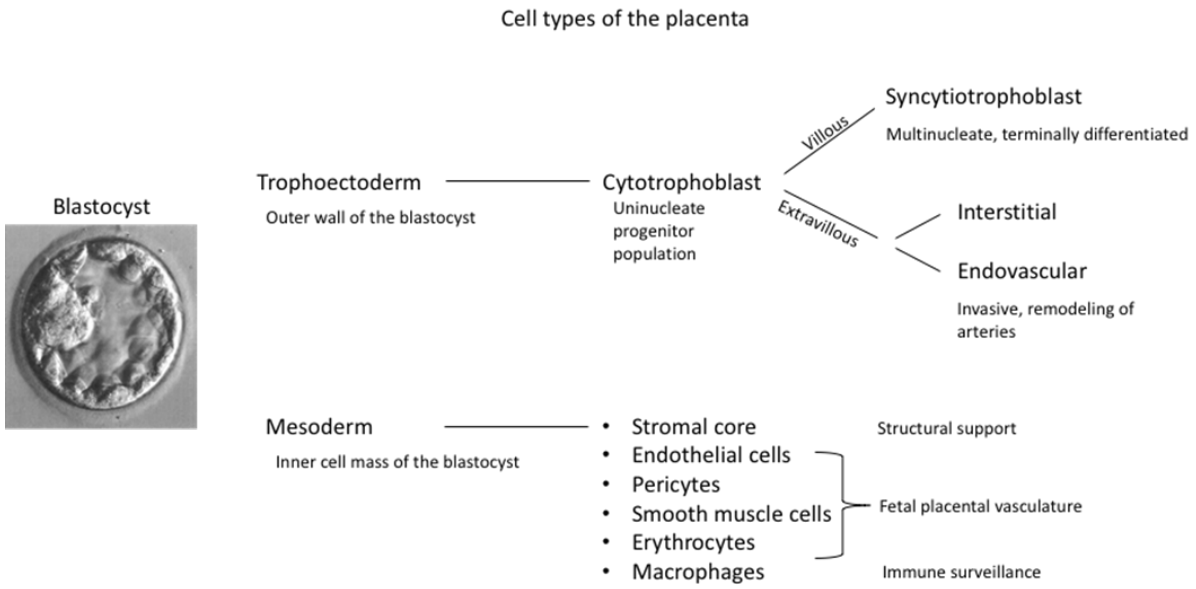

Figure 1.2 Cell types of the placenta, origins and differentiation. All cells of the placenta originate from the blastocyst. The outer wall of the blastocyst, or trophoectoderm, gives rise to the main functional component of the placenta, the cytotrophoblast. The extravillous cytotrophoblast can be categorised into the interstitial and intervascular component. The interstitial cells invade the maternal decidua, with a vast array of functions including regulation of immune-response. The endovascular cytotrophoblast invades maternal spiral arteries, remodelling them to connect to the fetal circulation. The villous syncytiotrophoblast is a multinucleate structure made up of fused cytotrophoblast cells surrounding the fetal villous tree, and are the main component regulating exchange between mother and fetus, as well as the main endocrine cell of the placenta.

The syncytiotrophoblast is the part of the placenta which carries the responsibility of forming the connection between fetal and maternal blood supplies by covering the chorionic villi and the entire surface of the growing placenta, and also the production of most of the placental hormones, such as β-hCG, estrogen and progesterone (Davies et al., 2016). The syncytiotrophoblast forms villi by forming invaginations that have a cytotrophoblast core, which the fetal mesenchyme will grow into forming secondary and finally tertiary villi composed of fetal capillaries (Gude et al., 2004). Meanwhile, the extravillous cytotrophoblasts form cell columns at the tips of chorionic villi between the fetal and maternal interface (Davies et al., 2016). Distally, these cytotrophoblasts cease to proliferate and penetrate the uterine wall into the maternal myometrium, anchoring the placenta to the myometrium and modifying maternal spiral arterioles to resist maternal vasomotor control (Davies et al., 2016; Winn et al., 2007). Extravillous cytotrophoblasts not only invade the maternal circulatory system of the myometrium, but invade interstitially as well (interstitial trophoblasts). By initiating and

promoting the expansion of the invaded area and by recruiting maternal arterioles, they allow the expansion of the underlying villous placenta (Gude et al., 2004). Tertiary villi differentiate from mesenchymal villi into immature intermediate villi (IIV), which branch into the stroma forming the villous tree of the mature placenta. The distal parts of the sprouting villi are mesenchymal villi, while their bases differentiate into IIV, which in turn differentiate into stem villi. In this way, 10 +/- 16 generations of stem villi form the villous tree found in the mature placenta (Kingdom et al., 2000).

1.2.1.3 Placental Vascularisation

During the first trimester of pregnancy, the placenta grows and differentiates in an hypoxic environment. The reason for this is that the spiral arterioles of the uterus are still partially blocked by the endovascular cytotrophoblast (Jauniaux, Gulbis, & Burton, 2003). At about 10-12 weeks of gestation, maternal blood finally begins to flow from the spiral arterioles into the intervillous space of the placenta (Gude et al., 2004; Jaffe, Jauniaux, & Hustin, 1997). The low oxygen environment is likely to be important for growth in the first trimester of development, since women who had premature blood flow into the intervillous space during the first trimester had a higher risk of miscarriage (Gude et al., 2004; Jauniaux et al., 2003). Blood vessel formation and vascularization of the human placenta begin at about 21 days, and the final growth phase of villous blood vessels takes place at the start of the third trimester at around 24 to 26 weeks, characterized by the capillaries growing longitudinally, coiling of capillaries and finally the formation of terminal villi (Gude et al., 2004). The fetoplacental capillaries are found within these terminal villi, and they are separated from maternal blood only by a thin layer of the vasculo-syncytial membrane, which is a thin layer of syncytiotrophoblasts (Kingdom et al., 2000). Subsequently, formation of terminal villi, dramatically increases the surface area to volume ratio for gas and nutrient exchange whereas the formation and growth of the vascular bed and blood vessels leads to an increase in fetal growth.

1.2.1.4 Placental transport

Although passive diffusion from the maternal blood stream to the fetus does take place, permeability of the placenta is quite low, so that the placenta must contain a multitude of specific transport systems in order to meet the growth and development needs of the fetus and resembles more the intestinal mucosa or renal epithelium than blood vessels (Stulc, 1997). The main substrate for fetal and placental metabolism is glucose, which crosses the placenta via facilitated diffusion (Burton & Fowden, 2015). It is probable that placental transport differs in early pregnancy and term pregnancy. This change may be regulated by oxygen tension and intervillous space blood flow (Glazier & Jansson, 2004; Gude et al., 2004). Any compound that crosses the maternal-fetal circulation must pass through the villous trophoblast, two membranes consisting of the microvillous maternal layer and the basal layer which faces the fetal blood (Haggarty, 2002). Respiratory gases can easily permeate the placental membrane, with the only rate-limiting factor being blood flow (Gude et al., 2004). Therefore, impairment of fetal or maternal blood flow can cause major changes in growth and development of the fetus (Burton & Fowden, 2015). Other compounds that pass through the placenta to the fetus do not simply permeate the placental membrane. The primary source of energy for the fetus, glucose, must come from the maternal circulation, and transport from mother to fetus is facilitated via protein-mediated diffusion and glucose transporters (GLUTs), mainly, GLUT3, GLUT4, GLUT8 and GLUT12 (Gude et al., 2004).

Amino acids, which can be metabolized by the fetal liver and are also required for protein synthesis, are transported via the microvillous membranes and syncytiotrophoblast. Since the ratio of amino acids between fetus and mother is greater than 1, it is probable that amino acids are transported via an active transport, energy consuming mechanism (Gude et al., 2004; Yudilevich & Barros, 1990). Because amino acids vary immensely in terms of structure, transporters of amino acids have relative specificity for different groups of substrates.

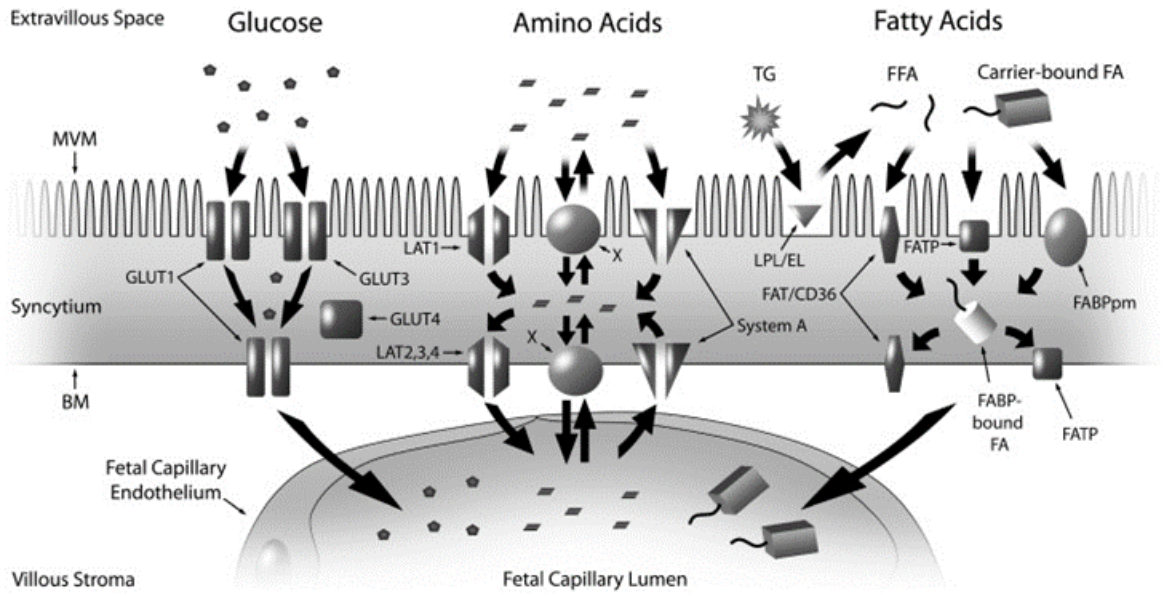

Figure 1.3 Placental transport across the placenta and the different types of transporters. MVM: Microvillous membrane; BM: basal membrane; GLUT: glucose transporter; LAT: large neutral amino acid transport; FAT/CD36: fatty acid translocase; LPL: lipoprotein lipase; EL: endothelial lipase; FATP: fatty acid transport protein; FFA: fatty acid; TG: triglycerides; FABP: fatty acid binding protein; FABPpm: plasma membrane fatty acid binding protein; X: exchangers. (Brett, Ferraro, Yockell-Lelievre, Gruslin, & Adamo, 2014).

Lipids are a heterogeneous group of compounds including phospholipids, glycolipids, triacylglycerols, free fatty acids, sphingolipids, cholesterol and fat-soluble vitamins, among others (Gude et al., 2004). Many lipids do not circulate freely, but are bound to plasma proteins, such as albumin and lipoprotein complexes. The maternal facing surface of the placenta therefore contains lipoprotein lipase, an enzyme that can release fatty acids from the lipoprotein complexes (Gude et al., 2004). Some lipids, such as free fatty acids and glycerol can cross the membranes of the syncytiotrophoblast via simple diffusion, which is important as fatty acids are needed by the fetus to develop and maintain membranes and as precursors of biologically active prostacyclins, prostaglandins, leukotrienes an thromboxanes, as well as using fatty acids as an energy source (Haggarty, 2002). Although fatty acids can permeate the membranes freely, fatty acid binding proteins have been identified, which probably facilitate and channel the uptake of free fatty acids (Haggarty, 2002). The placenta preferentially transports long chain polyunsaturated fatty acids, which are enriched in the fetal blood compared to maternal blood (Dutta-Roy, 2000; Gude et al., 2004) , as

they are required for structural lipid synthesis and essential for fetal development and cell function (Mennitti et al., 2015).

Water transfer and ion transfer across the placenta are closely linked, as transport of water depends on hydrostatic and osmotic gradients. Although water travels across the placenta passively, its transfer may be enhanced by water channel proteins expressed in the placenta (Gude et al., 2004; Stulc, 1997). The main extracellular cation is sodium (Na^+). Because of very steep electrochemical gradients across membranes, there is a strong driving force for substances to be transferred in co-transport with or in exchange for Na such as the Na^+/H^+ exchanger and trophoblast plasma membranes contain a multitude of systems that aid in transfer of nutrients to the fetus while also maintaining the right homeostasis in the cytosol of the trophoblast (Stulc, 1997). Chloride, being the main extracellular anion, can also enter cells via co-transport or specific chloride channels. Levels of phosphate, potassium and calcium are higher in the fetal blood. These ions permeate the placental membranes to the fetus via active transport, whereas sodium and chloride may also permeate via diffusion (Gude et al., 2004).

1.2.1.5 Placental hormones

One of the major functions of the placenta is to synthesise hormones and mediators, which are crucial for the successful outcome of pregnancy. These hormone expression levels change according to the stage of pregnancy, playing a role in the maintenance of pregnancy, the development of the fetus and finally the process of labour. (Costa, 2016). The main cell for hormone synthesis in the placenta is the syncytiotrophoblast, although other trophoblast phenotypes also produce some placental hormones (M. A. Costa, 2016; Lunghi, Ferretti, Medici, Biondi, & Vesce, 2007). One of the most important hormones produced in pregnancy is human chorionic gonadotropin (hCG). This hormone is produced mainly in the syncytiotrophoblast, though extra-villous trophoblasts may also synthesize hCG (Cole, 2012; M. A. Costa, 2016). hCG is measurable in maternal serum at 8 days post fertilization and is at its peak at 10 weeks of gestation (Cole, 2012; M. A. Costa, 2016) with another rise in late pregnancy (Gude et al., 2004). One of the main roles of hCG is to maintain early pregnancy by stimulating progesterone synthesis in the corpus luteum (Cole, 2012; Kelly et al.,

1991). Evidence also supports a role of HCG as a modulator of angiogenesis in the endometrium and placenta, also decreasing resistance in uterine arteries by increasing vasodilatatory and decreasing vasoconstrictive eicosanoids (Costa, 2016; Toth et al., 1994).

Progesterone is the hormone that maintains pregnancy and prevents early miscarriage, and studies have shown that up to 83% of all abortions have to do with low progesterone levels (Hahlin, Wallin, Sjöblom, & Lindblom, 1990; Tuckey, 2005). Once the syncytial layer is formed, progesterone is produced by the placenta, at around 6-8 weeks of pregnancy and increases until term when placental function is lost (Tuckey, 2005). Progesterone is a steroid hormone, and therefore produced from maternal cholesterol (Costa, 2016). In the placenta, progesterone is synthesized in a two-step process taking place in the mitochondria of the syncytiotrophobast (Costa, 2016). Progesterone exerts its effects in a non-genomic and genomic manner. Nuclear progesterone receptors modulate transcriptional events and are ubiquitously expressed in the placenta and female reproductive tract, while the non-genomic actions of progesterone are mediated via membrane progesterone receptors that rapidly activate pathways such as MAPK and genomic actions of progesterone enable implantation and attachment of the embryo (Costa, 2016; Zachariades et al., 2012). It also plays a role in immunotolerance, by enhancing profertility Th2 cytokines and promoting the differentiation of Th2 lymphocytes, as well as relaxation of the uterus (Costa, 2016; Raghupathy, Al Mutawa, Makhseed, Azizieh, & Szekeres-Bartho, 2005).

There are different types of placental estrogens, with the most abundant being 17ß-estradiol (Costa, 2016). 17ß-estradiol is produced exclusively in the corpus luteum during the first 5-6 weeks of pregnancy, with the placenta becoming the main source of the hormone after the first trimester (Tal, Taylor, Burney, Mooney, & Giudice, 2000). Concentrations of 17ß-estradiol increase gradually until term (Tal et al., 2000). Lacking enzymes to convert progestogens to estrogens, the placenta relies largely on dihydroepiandrostenediene sulfate (DHEAS) to produce estrogens (Costa, 2016; Tal et al., 2000). The effects of estrogens are mediated by activating nuclear receptors ERα and ERß, which dimerize and alter gene expression (Björnström & Sjöberg, 2005; Costa, 2016), as mentioned below. Estrogens can however also activate GPCRs such as GPR30, which activate non-genomic pathways such as increasing cAMP levels and

activation of the MAPK pathway (Björnström & Sjöberg, 2005; Costa, 2016). One of the main functions of estrogens during pregnancy is an increase of arterial blood flow (Resnik, Killam, Battaglia, Makowski, & Meschia, 1974; Tal et al., 2000). Other functions include regulating fetal progesterone production and secretion as well as the biosynthesis of placental progesterone, and also influencing the function of the mammary fetal adrenal gland (Tal et al., 2000).

1.2.2 Endocrine disrupting chemicals

1.2.2.1 Overview

Endocrine disrupting chemicals (EDCs) are a heterogeneous group of compounds found in the environment. They have the capacity to disrupt the physiological function of wildlife and humans and have recently taken centre stage as their effects are becoming more widely known. The U.S Environmental Protection Agency (EPA) defines an EDC as “an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process.” With the focus on industry and production in the 20th century, these synthetic chemicals were developed in order to advance science and agriculture, as well as industry and are now widely distributed throughout the environment via different routes such as chemical run-off. In 2012, the World Health Organization (WHO) stated in their State of the Science of Endocrine Disrupting Chemicals paper that three major concerns have highlighted reasons to investigate EDCs further (Bergman et al., 2013). Firstly, the paper states that there is a high incidence of endocrine disorders in humans as well as an increasing trend of these disorders. Secondly, these effects or similar have been identified in wildlife. Lastly, there have been increasing findings of endocrine disrupting chemicals linked to outcomes of disease in studies conducted in the lab (Bergman et al., 2013). With statements such as these, there has been an increased effort to elucidate ways in which EDCs affect the human body in early development, childhood, and adulthood.

1.2.2.2 Types of EDCs

There are hundreds of thousands of synthetic chemicals, and about 1000 of these have been identified as possibly having endocrine active properties (Gore et al., 2014). These chemicals can usually be organized into distinct groups, depending on their chemical structure and function, and also the role they play in industry. Polychlorinated biphenyls (PCBs) as well as polybrominated biphenyls (PBBs) are usually used as industrial solvents or lubricants, some, such as bisphenol A (BPA) and phthalates are used as plasticizers, others are used in agriculture such as the pesticides methoxychlor, dichlorodiphenyltrichloroethane (DDT) and chlorpiryfos. Pharmaceutical agents such as the xenoestrogen dietholstilbestrol (DES), which was previously prescribed as an anti-abortive agent until it was found to cause vaginal clear-cell adenocarcinoma in female offspring of women who had taken DES during pregnancy, among many other health risks, have been used in the past and now (Diamanti-Kandarakis et al., 2009; Giusti, Iwamoto, & Hatch, 1995). Even some naturally occurring chemicals have the capacity to disrupt the endocrine system, especially when consumed in large amounts and during crucial windows of development. Phytoestrogens, which include chemicals such as genistein and dadzain, are widely found in soy milk and used in infant formula (Diamanti-Kandarakis et al., 2009; Harlid et al., 2017).

Sources	Category	Substances
Incineration, landfill	Polychlorinated compounds	Polychlorinated dioxins, polychlorinated biphenyls
Agricultural runoff/atmospheric transport	Organochlorine pesticides	DDT, dieldrane, lindane
Agricultural runoff	Pesticides currently in use	Atrazine,trifluraline, permethrin
Harbours	Organotins	Tributyltin
Industrial and municipal effluents	Alkylphenols	Nonylphenol
Industrial effluent	Phthalates	Dibutyl phthalate, butylbenzyl phthalate
Municipal effluent Agricultural runoff	Natural hormones; synthetic steroids	Estradiol, estrone, testosterone, ethinyl estradiol
Pulp mill effluents	Phytoestrogens	Isoflavones, lignans, coumestans
Consumer products	Cosmetics, personal care products, cleaners, plastics	Parabens, phthalates, glycol ethers, fragrances, cyclosiloxanes, bisphenol A (BPA)

Table 1.1 Types of EDCs and their sources. Adapted from: Dodson et al. (2012). EDCs. EDCs have a multitude of man-made sources such as incineration and landfill, runoff from agriculture, effluents from industry, harbours, municipal effluents, effluents from pulp mills, as well as consumer products such as cosmetics, personal care products, cleaners, plastics and medication. They can be categorised into polychlorinated compounds, organochlorine pesticides, other pesticides, organotins, alkylphenols, phthalates, natural hormones, synthetic steroids and phytoestrogens.

1.2.2.3 Polychlorinated biphenyls

Polychlorinated biphenyls (PCBs) were historically used in industry as coolants or in electrical transformers, as well as microscope immersion oils, copy paper, or in pesticides, and in many other industrial applications involving heat transfer, as plasticizers, rubber products, pigments and dyes among others, as they had favourable properties such as non-flammability and chemical stability (Crinnion, 2011; Faroon & Ruiz, 2015). Although PCBs are no longer produced in most countries due to their known toxicity (Quinete, Esser, Kraus, & Schettgen, 2017), PCBs are still released into the environment from hazardous waste sites, improper dumping of waste that contains PCBs, leaks from products such as transformers still containing PCBs, and wrongful disposal of PCB-containing products (Agency for Toxic Substances and Disease Registry (ATSDR), 2000).

Furthermore, PCBs bioaccumulate in the food chain, preferentially in fatty tissues due to their lipophilicity and can therefore persist (Faroon & Ruiz, 2015; Ivanciuc, Ivanciuc, & Klein, 2006). A study from 2001 estimated mean daily intake of dioxin and dioxin-like compounds, which include PCBs, to be 2.4 pg/kg/boy weight in male adults and 2.2 pg/kg/body weight in female adults (Schecter et al., 2001).

PCBs have been linked to a multitude of pathologies in humans and wildlife, ranging from developmental pathologies, endocrine pathologies, systemic effects, immunological, neurological and reproductive effects, as well as being linked to some cancers. In rats, PCB treatment was linked to higher cholesterol levels, increased blood pressure, as well as an increase in myocardial mass (Lind, Örberg, Edlund, Sjöblom, & Lind, 2004). In keeping with these findings, PCBs were found to be associated with hypertension in humans (Peters, Fabian, & Levy, 2014; Yorita Christensen & White, 2011). Often found in combination with hypertension, type 2 diabetes was also found to be associated with PCB exposure in humans (Airaksinen et al., 2011; Codru et al., 2007; Everett et al., 2007; Kim et al., 2014). As well as affecting the body systemically, PCBs have been shown to affect neurological development and behaviour in rats (Coburn, Gillard, & Currás-Collazo, 2005; Cromwell et al., 2007; Dziennis et al., 2008; Pruitt, Meserve, & Bingman, 1999;

Simmons, Cummings, Clemens, & Nunez, 2004) and on neurodegenerative disease such as Parkinson's disease, dementia and amytrophic lateral sclerosis (ALS) in women (Steenland et al., 2006).

Importantly, PCBs have been found to play a role in human development in different phases of life as well as having an effect on human reproduction. Developmentally, PCB exposure has been associated with cryptorchidism in males when exosed *in utero* (Brucker-Davis et al., 2008), as well as impaired visual evoked potentials (VEPs) in 15 month old children who had higher exposures to PCB congeners during breastfeeding (Riva et al., 2004). Furthermore, increased PCB ingestion in girls was associated with earlier onset of menarche, with a median age of 12.2 years (Denham et al., 2005) and higher serum levels of PCBs were associated with polycystic ovarian syndrome in Chinese women (Yang et al., 2015) . In males, PCBs negatively affected sperm motility (Jiang et al., 2017) and was associated with lower androgen levels (Vitku et al., 2016).

1.2.2.4 Phthalates

Phthalates include phthalic acid and all phthalic acid diesters and are a group of chemicals that are mainly produced for the manufacturing of polyvinyl chloride plastics (PVC) (Benjamin et al., 2017) but they can also be found in tablecloths, tiles, upholstery, walls, shower curtains, carpets, garden hoses, pesticides, toys and cosmetic products (Biomonitoring Phthalates, Americas Children and the Environment, USEPA, 2017). As phthalates are still being commonly used in all aspects of industry, despite evident concerns about their safety, exposure to phthalates is ubiquitous. Phthalates have been measured in many human tissues and fluids including blood, urine, breastmilk, semen and placenta (Berger et al., 2018; Chang, Wu, Pan, Guo, & Lee, 2017; Duan et al., 2017; Fromme et al., 2011; Machtinger et al., 2018). The most common route of exposure in humans is oral ingestion, with inhalation and skin exposure also being important (Kimber & Dearman, 2010). There have been a multitude of studies concerned with the health effects of all different kinds of phthalates, ranging from exposure during pregnancy, infancy, childhood, adolescence and adulthood. As with most EDCs, a large amount of associations between phthalate exposure and disease have been found in every stage of life.

In pregnancy, higher concentrations of phthalates were associated with higher BMI and lower birth weight (Bellavia et al., 2017), and *in utero* exposure to phthalates in the 3rd trimester was associated to earlier pubertal onset in girls, indicative of acting on the gonadal axis (Watkins et al., 2017). There is also a relationship between elevated phthalate levels in the urine of pregnant women and occurrence of preterm birth (Ferguson, Mcelrath, Ko, Mukherjee, & Meeker, 2014). Prenatal exposure to phthalates also has been shown to be associated with adverse outcomes in cognition and behaviour in children including attention deficits, hyperactivity, lower IQ and poorer communication skills (Ejaredar, Nyanza, Eycke, & Dewey, 2015; Engel et al., 2010; Kim et al., 2009; Kim et al., 2011; Miodovnik et al., 2010; Swan et al., 2010; Téllez-Rojo et al., 2013; Whyatt et al., 2011; Yolton et al., 2011).

Pre-pubertal exposure to phthalates has also been associated with altered pubertal timing in girls (Kasper-Sonnenberg, Wittsiepe, Wald, Koch, & Wilhelm, 2017).

Furthermore, increased urinary phthalates were associated to both higher insulin resistance and higher body weight in children (Benjamin et al., 2017; Teitelbaum et al., 2012; Trasande, Spanier, Sathyanarayana, Attina, & Blustein, 2013; Trasande, Attina, Sathyanarayana, Spanier, & Blustein, 2013). In adults, increased phthalate levels were associate with cardiovascular effects such as heart-rate variability, obesity, type 2 diabetes, hypertension, upregulation of low grade inflammatory biomarkers and altered circulatory thyroid hormone levels (Bai et al., 2017; Dong et al., 2017; Jaimes et al., 2017; Park et al., 2017).

1.2.2.5 Dichlorodiphenyltrichloroethane (DDT)

DDT is the most widely known organochlorine insecticide and was developed in the 1930s. It was used all throughout the 20th century as a pesticide and also to eradicate malaria, and it was banned in the U.S in the 1970s and in the U.K in 1986, due to its neurotoxic properties (Costa, 2015). Although the use of DDT has been discontinued in most countries, the bioaccumulation of the lipophilic compound and biomagnification in the food chain has caused the pesticide to still be relevant today, as humans are still commonly exposed to it mainly via their diets, although respiratory and dermal intake also play a minor role (Mansouri et al., 2017). It has been therefore classified as a persistent organic pollutant (POP). DDT and its derivates are stored in adipose tissue, and are excreted via the bile, urine and breast milk (Costa, 2015; Pirsaheb, Limoee, Namdari, & Khamutian, 2015) and have also been detected in the placenta (Toichuev et al., 2017). The neurotoxic effects of DDT have been known since at least the 1970s, and acute exposure to high doses of the substance can cause motor unrest and increase of spontaneous movements, increased susceptibility to fear and external stimuli, tremors progressing to tonic-clonic seizures, as well as death due to respiratory failure (Costa, 2015).

A multitude of studies in recent years have shown that neurotoxicity is not the only adverse effect DDT is related to. As with other EDCs, DDT can have *in utero* effects that manifest later in life. This is supported by a study that related obesity in 12 year old boys with DDT exposure *in utero* (Warner et al., 2017) as well as a study that found changes in sex hormone levels of 12 year old boys (Eskenazi et al., 2017) after being exposed to DDT *in utero.* A study also found that DDT exposure *in utero* was related

to higher body weight in girls aged 1-2 years (Coker et al., 2018) Furthermore, higher levels of cord blood DDT in newborns were associated with smaller head circumference (Arrebola, Cuellar, Bonde, González-Alzaga, & Mercado, 2016).

In adults, DDT exposure has also been associated to various diseases. In men, exposure to DDT was associated with lower sperm quality, motility, concentration and altered morphology (Aneck-Hahn, Schulenburg, Bornman, Farias, & De Jager, 2006; Messaros et al., 2009). DDT was also associated with an increased rate of metabolic syndrome among adults including higher rates of hypertension, central obesity, dysglycemia and dyslipidemia (Rosenbaum, Weinstock, Silverstone, Sjödin, & Pavuk, 2017) and type 2 diabetes (Evangelou et al., 2016) as well as impaired lung function (Ye, Beach, Martin, & Senthilselvan, 2016). In women, DDT has been associated with hypertensive disorders in pregnancy (Murray et al., 2018) as well as polycystic ovarian syndrome (Guo et al., 2017).

1.2.2.6 Phytoestrogens

Phytoestrogens, classified into isoflavones, coumestans and lignans, are a group of nonsteroidal xenoestrogen found throughout the plant kingdom that have the potential to exert effects via their estrogenic function and therefore can be classified as EDCs. Although generally phytoestrogens have been studied for their health benefits, such as possible protective functions against cancer, atherosclerosis and osteoporosis (Patisaul & Jefferson, 2010) recent studies have been emerging that highlight less beneficial and possibly harmful effects of phytoestrogens such as younger age of menarche and a slower waning of infantile breast tissue in girls (Marks et al., 2017; Zung, Glaser, Kerem, & Zadik, 2008), uterine fibroids (D'Aloisio, Baird, DeRoo, & Sandler, 2010), Kawasaki disease (Portman, Navarro, Bruce, & Lampe, 2016), cardiovascular disease (Reger, Zollinger, Liu, Jones, & Zhang, 2016), and a reduction in chemotherapy sensitivity (Belcher et al., 2017) . Although the data on these compounds is often conflicting, it is important to note that any compounds that mimic hormones can have negative effects on the human body, although this is largely influenced by amount ingested, stage of human development, gender, and exposure to other EDCs which could potentiate this effect. Phytoestrogens are found in various plants such as berries, seeds, legumes fruits and nuts, but are most highly

concentrated as isoflavones genistein and dadzain in soy beans and soy products (Patisaul & Jefferson, 2010).

1.2.2.7 Bisphenol A (BPA)

Bisphenol A (BPA) is a compound first synthesized in 1891 (Tyl, 2014) which is now used in a variety of products such as plastics, the lining of aluminum cans and thermal receipts (Peretz et al., 2014) and is one of the emerging pollutants most frequently detected in the environment (Muhamad, Salim, Lau, & Yusop, 2016). BPA is an EDC and a xenoestrogen which interacts with estrogen receptors ERα and ERß, membrane bound estrogen receptors such as GPR30 and other receptors such as the thyroid hormone receptor (Peretz et al., 2014; Richter et al., 2007).

Although BPA clearly shows estrogen-mimicking activity, BPA can also act as a selective estrogen receptor modulator (SERM) in the mouse uterus, and can inhibit androgen activity as well as having an anti-thyroid hormone effect, though these inhibitory effects at androgen and thyroid receptors occur at higher doses than estrogenic and anti-estrogenic effects (Lee, Chattopadhyay, Gong, Ahn, & Lee, 2003; Nagel, Hagelbarger, & McDonnell, 2001; Richter et al., 2007; Welshons, Nagel, & Vom Saal, 2006). Structurally, BPA consists of a phenolic and hydroxyl group bound to an aromatic ring, which can bind to other compounds to form polymers when used in the manufacturing process (Erler & Novak, 2010; Michałowicz, 2014; Muhamad et al., 2016). Conditions such as heat or acidic or basic environments can cause leaching of BPA into its surroundings, leading to potential environmental and human exposure and health risks, with exposure to BPA being predominantly oral (Richter et al., 2007; Srivastava, Gupta, Chandolia, & Alam, 2015). Infants aged 0-6 months exclusively fed with canned liquid formula and using polycarbonate bottles have been estimated to have highest BPA exposures (Srivastava et al., 2015).

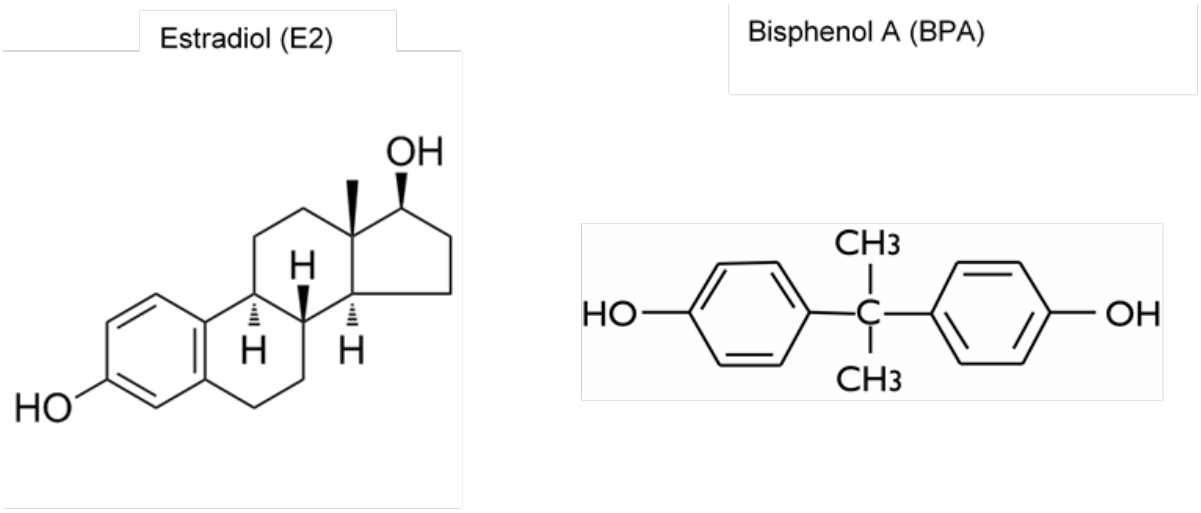

Figure 1.4 Similarity between BPA and E2. Estradiol is a type of estrogen and is an estrane steroid with 2 hydroxyl groups. BPA is a diphenylmethane derivative with two hydroxyphenyl groups.

1.2.2.7 Windows of exposure to EDCs

The idea that adult diseases can be caused by defects in development occurring *in utero* was developed in the late 1980s and throughout the 1990s (Barker Hypothesis) (Barker, 1992; Barker, 1997). Although EDCs can be harmful to human health when humans are acutely exposed to the chemical, and large quantities of EDCs can be immediately harmful, concerns have been raised about the delayed effects of EDCs, especially when the exposure takes place during a developmentally vulnerable time, i.e. during development *in utero* or in infancy. These early developmental windows are particularly vulnerable to harmful effects of EDCs because they occur when organogenesis and the development of tissues occur, and these events are controlled by finely regulated molecular and biochemical processes (Prusinski, Al-Hendy, & Yang, 2016).

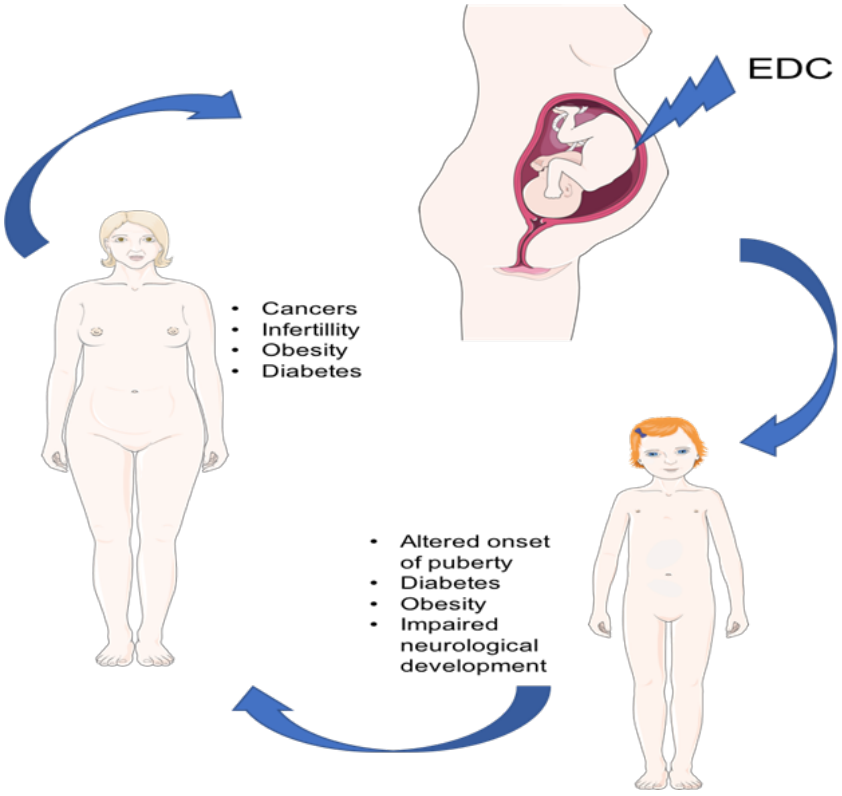

Figure 1.5 EDC exposure in *utero* can lead to diseases and developmental problems later in life, such as in childhood, puberty or adulthood. Fetal EDC exposure has been shown to lead to altered onset of puberty in offspring, as well as ovarian pathologies such as PCOS which can lead to fertility later in life. Furthermore, EDC exposure can be linked to the development of obesity in childhood and/or adulthood, further exacerbating diseases such as type 2 diabetes, and increasing proneness to certain cancers, whereas studies have found direct causal links between certain EDCs and the development of sometimes rare cancers in adulthood.

Historically, it was found that the prescription of DES to pregnant women led to their daughters developing rare reproductive cancers and higher rates of breast cancer, as well as complications during pregnancy or even spontaneous abortion (Reed & Fenton, 2013). Because it is difficult to retrospectively identify exposures *in utero*, most studies concerning exposures in the womb and development of health problems later in life have been conducted on animals.

Studies in rats have found that exposure to EDCs while pregnant can disturb follicle generation in the offspring as well as altering ovarian gene expression, factors which can lead to infertility later in life (Lawson et al., 2011; Santamaría, Durando, Muñoz De Toro, Luque, & Rodriguez, 2016; H.-Q. Zhang et al., 2012). Mixtures of EDCs given *in utero* were found to decrease rat follicle reserves and to accelerate reproductive ageing (Johansson et al., 2016). In keeping with EDCs potentially causing aberrant

development of the ovaries, the exposure *in utero* of different species to androgens has been linked to polycystic ovarian syndrome (PCOS). In humans, increased maternal testosterone levels at 18 weeks pregnancy have been linked to increases of anti-Mullerian hormone (AMH) levels in female offspring of those mothers during adolescence, which increases the risk of developing PCOS later in life (Hart et al., 2010; Hewlett, Chow, Aschengrau, & Mahalingaiah, 2017). In rodents, phthalate exposure *in utero* was linked to the development of phenotypes related to PCOS later in life (Manikkam, Tracey, Guerrero-Bosagna, & Skinner, 2013). Finally, fetal exposure to DDT predicted an increased risk of breast cancer in adulthood when controlling for maternal lipids, rage, age weight and breast cancer history (Cohn et al., 2015; Mallozzi, Bordi, Garo, & Caserta, 2016).

1.2.3 Estrogen receptor structure and function

1.2.3.1 Overview

Estrogens are crucial hormones in the human body, regulating many vital processes such as cell growth, cell reproduction, and development and differentiation. Estrogen is an important sex hormone, which regulates aspects such as growth, physiology and development of the human reproductive system, and also plays a role in the development and function of the cardiovascular, skeletal and neuroendocrine system (Lee, Kim, & Choi, 2012). Moreover, it regulates sympathetic activation, inflammation, glucose metabolism, and lipid metabolism (Barton, 2016). The predominant estrogen found in the body, 17ß-estradiol (E2) is primarily synthesized in the ovary of premenopausal women, whereas in postmenopausal women and in men, E2 is synthesized in extragonadal tissues such as the brain and adipose tissue (M. Jia, Dahlman-Wright, & Gustafsson, 2015). Estrogen receptors α and β (ERα and ERβ) can act as transcription factors that are activated through the binding of estrogen, after which they bind to DNA within target gene promoters via their Estrogen Response Elements (EREs). ERα and ERβ have different and overlapping functions and are often found in different tissues(Lee et al., 2012).

1.2.3.2 Estrogen receptor structure

Like most other nuclear receptors (NRs), ERs contain distinct domains that are structurally and functionally conserved (Heldring et al., 2007). ERs consist of the DNA binding domain (DBD), the COOH- terminal ligand-binding domain (LBD) and the NH_2-terminal domain which is the most variable domain. Activation functions (AF) are located at the DBD and LBD and recruit coregulatory proteins to the receptor when bound to DNA (Heldring et al., 2007). The two ERs have a similar affinity to E2 and also bind to the same DNA elements, though they are coded by different genes which are located on separate chromosomes (Heldring et al., 2007).

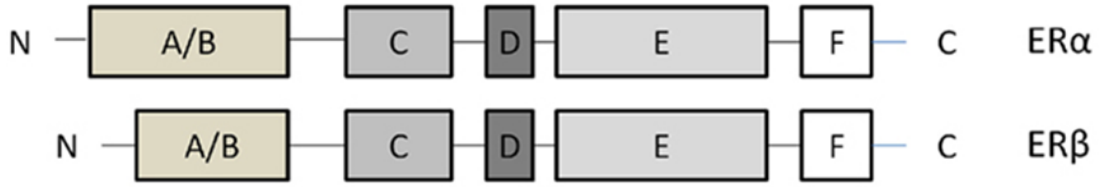

Figure 1.6 Structure of estrogen receptors. Each contain the domains A-F. A/B: transactivation mediation in the absence of ligand. C: Binding sites of EREs. D: hinge region. E and F: estrogen and estrogenic compound binding sites (Karimian, Chagin, & Sävendahl, 2011).

1.2.3.3 Estrogen receptor signaling

There is a high order of complexity of ER signaling. Upon ligand binding, ERs dimerise, translocate to the nucleus and bind to specific estrogen response elements (ERE) on DNA promoter regions, where they can also interact with other transcription complexes, thereby influencing the transcription of genes unspecific for the binding of ligand bound ERs (Heldring et al., 2007). ERs can also be phosphorylated by activated kinases -in a ligand independent fashion-, dimerise and regulate gene transcription as well, which can be seen in certain hormone independent tumours (Kampa, Pelekanou, Notas, Stathopoulos, & Castanas, 2013).

ERs can also exert effects via different pathways, apart from the canonical nuclear signaling (Kampa et al., 2013). E2 can bind to membrane-bound ERα stimulating G-proteins and processes such as inositol phosphate generation, Ca^{2+} influx in to the cell, activation of phospholipase C, the ERK/MAPK pathway and activation of the

P13K/AKT pathway within minutes or even seconds of binding (Marino, Ascenzi, & Acconcia, 2006). ERα and ERß can become membrane bound through a process called palmitoylation and other lipid modification mechanisms, where they can exert the previously mentioned non-genomic functions (Meitzen et al., 2013).

Furthermore, non-genomic pathways activated by estrogens can also be mediated via the membrane-bound g-protein coupled receptor GPR30, or GPER. GPR30 is a GPCR discovered in 1996 (Owman, Blay, Nilsson, & Lolait, 1996; Revankar, Cimino, Sklar, Arterburn, & Prossnitz, 2005) and binds estrogen with an affinity 10x higher than ERα (Filardo, Quinn, Bland, & Frackelton, 2000) . GPR30 plays a role in the physiology of the reproductive system, endocrine metabolism, and adipocyte function, as well as being involved in reproductive cancers, male fertility, osteoporosis, obesity, hypertension, auto-immune diseases and ageing. It is also responsible for abnormal estrogen mediated vasoconstriction and elevated blood pressure in female mice (Barton, 2016). Importantly, numerous endocrine disrupting chemicals, including atrazine, BPA, chlorinated hydrocarbons and nonylphenol have been found to activate GPR30 due to their xenoestrogenic characteristics (Barton, 2016; Prossnitz & Arterburn, 2015). In the case of BPA, it has been shown to bind to GPR30 with a similar affinity as E2 (Alonso-Magdalena et al., 2005; Nadal et al., 2000).

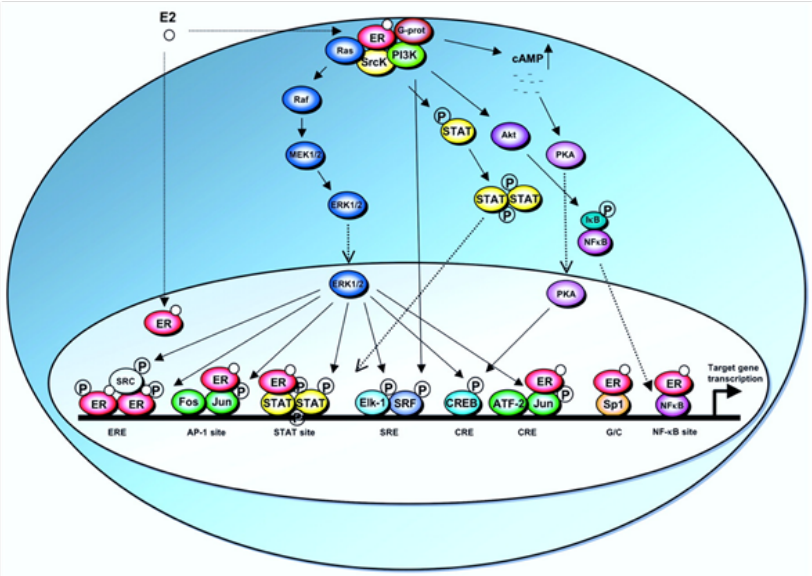

Figure 1.7 Interplay of estrogen receptor nuclear and non-nuclear receptor signaling (Björnström & Sjöberg, 2005). Classically, ERs are nuclear receptors that bind to estrogen response elements (EREs), in order to affect cellular physiology via gene regulation. More recently, estrogenic compounds have been found to also bind to membrane-bound receptors, such as GPR30 or membrane bound ERα or ERβ. Mechanisms of action are mediated via intracellular pathways activating intracellular targets such as P13K, MAPK and AKT.

1.2.4 Bisphenol A and its effects on pregnancy, development, and female reproduction

1.2.4.1 Health risks associated with BPA

A multitude of health risks have been implied in relation to BPA exposure in humans, from cancers to developmental abnormalities, fertility problems and metabolic diseases to name a few. Studies have shown that BPA has neurobehavioral, neurotoxic, and neuroendocrine effects (Srivastava et al., 2015). In both males and females, changes in hypothalamus-pituitary-gonadal axis were induced by BPA and prolonged estrous cycles were observed in mice (Rubin, Murray, Damassa, King, & Soto, 2001; Srivastava et al., 2015). Furthermore, BPA exposures during development affect brain structure and function as well as behavior in mice (Richter et al., 2007). BPA has the capacity to contribute to metabolic diseases such as diabetes mellitus and obesity, with BPA accumulating in adipose tissue and increasing the number and

size of adipocytes, thereby contributing to weight gain (Srivastava et al., 2015). Furthermore, BPA exposure has been linked to carcinogenicity, especially of the prostate and mammary gland (Cheong et al., 2016; Marchese & Silva, 2012). Prenatal BPA exposure may influence the development of prostate cancer in later life and also may increase the frequency of mammary tumours through either alteration of fetal glands or by mediating estrogen-dependent growth of tumour cells (Srivastava et al., 2015). In terms of male and female fertility, BPA has been linked to various effects on the reproductive system. In men, high concentrations of BPA lowered sperm motility and kinematics as well as causing poor fertilization (Rahman et al., 2015). Furthermore, men with higher urinary BPA concentrations due to work exposure reported more sexual dysfunction, reduced sex drive and erectile dysfunction than unexposed men (Cantonwine, Hauser, & Meeker, 2013; D. Li et al., 2010). In females, studies have shown an increase in infertility due to BPA exposure caused by disruption of oocyte maturation and E2 production in the ovary, as well as early pregnancy loss (Cantonwine et al., 2013). In a population of women undergoing IVF, a positive correlation between BPA concentrations in urine and odds of implantation failure was described (Cantonwine et al., 2013; Ehrlich et al., 2012). Takeuchi et al found a strong relationship between serum BPA and androgen concentrations, possibly providing a link to ovarian dysfunction such as PCOS (Takeuchi, Tsutsumi, Ikezuki, Takai, & Taketani, 2004).

1.2.4.2 BPA metabolism and concentrations

In the human body, BPA has been detected in fluids such as fetal and maternal plasma, amniotic fluid and follicular fluid. It has also been detected in the placenta (Balakrishnan, Henare, Thorstensen, Ponnampalam, & Mitchell, 2010). Variable concentrations have been found in these compartments, ranging from 0.3 to 18.9 ng/mL in the maternal plasma and 0.2 to 9.2 ng/mL in the fetal plasma, as well as 1.0 to 104.9 ng/g in term placenta in humans (Balakrishnan et al., 2010; Schönfelder et al., 2002). The major part of ^{14}C-BPA (^{14}C-labeled BPA) is excreted in feces and urine, with the major urine metabolite was determined to be ^{14}C-BPA-monoglucuronide (Pottenger et al., 2000; Schönfelder et al., 2002). BPA-monoglucuronide is the biologically inactive form of BPA (Trdan Lušin, Roškar, & Mrhar, 2012), and rapid conversion to this form after oral administration results in low BPA bioavailability.

However, studies have shown that very low levels of BPA can alter the development of reproductive organs, such as the mammary gland and the vagina in mice (Schönfelder et al., 2002). After administering a single oral dose of BPA, conjugation is rapid and almost complete after administration, with less than 1% of total BPA being unconjugated and that elimination of these conjugates in the urine occurs largely within 24 hours (Thayer et al., 2015). However, biovailability in rats depends on the administration route, with the lowest bioavailibilty resulting from oral administration, while subcutaneous or intraperitoneal doses resulted in higher bioavailibilities (Pottenger et al., 2000). Interestingly, in models of placental perfusion, there was a gradual decline of BPA in the maternal compartment and a simultaneous increase in BPA concentrations in the fetal compartment, showing that BPA can cross the placenta, even at low levels (Balakrishnan et al., 2010). Confirming this, Ikezuki et al found BPA to be present in the amniotic fluid at levels that are five-fold higher than those found in the corresponding maternal sera (Ikezuki et al., 2002), indicating a possible active transport of BPA across the placenta.

1.2.4.3 BPA and its effects on pregnancy, development and the placenta

Studies have shown that BPA has effects on human pregnancy and the placenta, resulting in conditions that could be harmful to both mother and child and potentially influencing the development of the fetus and causing problems later in life. There is a correlation between BPA exposure *in utero* and implantation problems, as well as pathological pregnancies, pre-term births and low birth weight. Furthermore, BPA exposure has been linked to changes in fetal development concerning a vast variety of organs and systems in utero. Not only does BPA have the potential to disrupt or alter development of the fetus, but -as mentioned previously- exposure to BPA during development inside the womb can also lead to health issues in later life. Furthermore, structure and function of vital organs during pregnancy such as the placenta can be altered, adding to or causing effects that may result in pathological pregnancies, IUGR and even early and late miscarriage.

One pregnancy related complication BPA has been linked to is preeclampsia. Preeclampsia (PE) is a pregnancy disorder that is defined by a newly diagnosed hypertension and proteinuria or, in the absence of proteinuria thrombocytopenia, renal

insufficiency, impaired liver function, pulmonary oedema or visual or cerebral problems (Gathiram & Moodley, 2016). PE is one of the principal causes of maternal mortality, and is associated with health risks that develop later in life such as obesity and type II diabetes (Gathiram & Moodley, 2016; O'Tierney-Ginn & Lash, 2014). Because the main characteristic of (early-onset) PE is inadequate spiral artery transformation, resulting in placental hypoperfusion and therefore inadequate supply of nutrients to the fetus, fetal growth restriction(FGR) or IUGR is often the result (Gathiram & Moodley, 2016). Urinary concentrations of BPA measured in pregnant women were significantly associated with an increased risk of preeclampsia, thus demonstrating a positive correlation between elevated levels of PBA and PE (Cantonwine et al., 2016). Moreover, normotensive women had significantly lower BPA levels in the placenta than women with preeclampsia (Leclerc, Dubois, & Aris, 2014). BPA was also associated with an increased fms-like tyrosine kinase-1 (sFlt-1, or VEGFR-1) to placental growth factor (PlGF) ratio, and that lower levels of circulating PlGF and higher levels of sFlt-1 in pregnancy are associated with a higher risk of the development of diseases related to altered trophoblast function, such as PE (Ferguson, McElrath, Cantonwine, Mukherjee, & Meeker, 2015; Widmer et al., 2007). Detrimental effects on cytotrophoblasts such as apoptosis and necrosis due to low doses of BPA were observed by Benachour and Aris, and the authors postulated an outcome *in vivo* leading to pathologies such as PE, adverse pregnancy outcomes, preterm birth, IUGR and pregnancy loss (Benachour & Aris, 2009).

Not only does BPA increase the risk of adverse effects in pregnancy, but studies have shown that BPA is linked to various effects inhibiting the onset of pregnancy all together. Firstly, there may be a correlation between BPA exposure and infertility. Brieño-Enriquez et al showed that BPA exposure in early stages of meiosis can result in higher incidences of crossing-over and an increase of oocyte degeneration (Brieño-Enríquez et al., 2011). In late meiosis, BPA exposure can result in an increase of meiotic abnormalities and aneuploidy in oocytes (Hodges et al., 2002; Machtinger & Orvieto, 2014). Another study reported meiotic spindle alterations, chromosomal malalignment, an increase in meiotic arrest and aneuploidy were all associated with exposure to BPA in the final stages of meiosis (Hunt et al., 2003; Machtinger & Orvieto, 2014). BPA exposure has been linked with impairment of follicular development (Machtinger & Orvieto, 2014; Peretz, Gupta, Singh, Hernández-Ochoa, & Flaws,

2011), as well as epigenetic changes such as alterations in DNA-methylation and histone modification, leading to meiotic errors, which could inhibit pregnancy (Machtinger & Orvieto, 2014; Trapphoff, Heiligentag, El Hajj, Haaf, & Eichenlaub-Ritter, 2013) .

Besides infertility inhibiting the onset or development of pregnancy, another major factor that regulates development of a viable pregnancy is implantation. Improper implantation of the embryo leads to insufficient development of the fetal-maternal interface, making the further development of pregnancy impossible. Treating mice with BPA lead to “improper endometrial epithelial and stromal functions,” thereby being detrimental to embryo implantation and subsequent establishment of gestation. This effect was brought on through BPA affecting the PGR-HAND2 pathway (Li et al., 2016). A series of studies produced by Berger et al found that administration of BPA disrupted normal implantation in mice and resulted in a decrease of implantation sites. Furthermore, exposure to BPA in the first four days of pregnancy reduced litter size significantly and was associated with a significant reduction of pregnancies (Berger, Foster, & deCatanzaro, 2010; Berger, Hancock, & deCatanzaro, 2007; Berger, Shaw, & deCatanzaro, 2008; Machtinger & Orvieto, 2014). BPA treatment of pregnant mice resulted in a complete absence of implantation sites, delayed implantation and increased perinatal mortality, possibly due to the direct disruption of uterine receptivity towards blastocyst implantation due to the estrogenic properties of BPA (Machtinger & Orvieto, 2014; Xiao, Diao, Smith, Song, & Ye, 2011). More recently, it has been shown that high doses of BPA significantly decreased expression of the adhesion proteins ß3 and trophinin in the endometrium and in blastocysts, resulting in adhesion failure of the blastocyst and thereby failure of implantation in mice (Pan et al., 2015).

The development of the fetus during pregnancy or of the offspring at birth or in later developmental stages in life has also been studied in the context of BPA exposure. There is a potential relationship between BPA exposure and low birth weight of infants, especially female (Huo et al., 2015). Besides affecting birth weight, high doses of BPA can affect growth of offspring in the first years of life (Guida et al., 2015; Morrissey et al., 1987). Low dose exposure to BPA, more representative of actual human exposures can also induce behavioral and neuronal alterations and cognitive deficits (Guida et al., 2015). In general, fetal malformation was higher in offspring from

mothers with higher levels of free circulating BPA (Guida et al., 2015). BPA exposure *in utero* can also cause disruptions of normal "sexually dimorphic behaviours" later in life, such as exploration, social interactions, and sexual and parental behaviour, affecting males and females and different species differently (Palanza, Nagel, Parmigiani, & Vom Saal, 2016). Furthermore, pregnant mice that were exposed to BPA in the range of human exposure showed increases in prostate volume and a decrease in sperm production of adult male offspring (vom Saal et al., 1997; Vom Saal et al., 1998; Witorsch, 2002). Another study showed a relationship between low-dose *in utero* BPA exposure with an increased body weight of prepubertal male and female mice (Howdeshell, vom Saal, Hotchkiss, Thayer, & Vandenbergh, 1999; Witorsch, 2002). Prenatal exposure to BPA is associated with disruptions in organ development in mice such as lung (Hijazi, Guan, Cernea, & Yang, 2015), liver (DeBenedictis, Guan, & Yang, 2016) and mammary gland (Grassi et al., 2016) alterations. Several studies, such as a study by Hoepner et al, have found that BPA exposure might be associated with adiposity in childhood (Hoepner et al., 2016) and later in life. Furthermore, a relationship between low dose BPA exposure during critical developmental periods including fetal development and the development of metabolic diseases including type II diabetes has been documented (Alonso-Magdalena, Quesada, & Nadal, 2015).

1.2.5 Placental modeling using animal models, 2D human tissue culture and 3D human tissue culture

1.2.5.1 Overview

Understanding placental physiology in humans is crucial in order to develop reliable models to test placental function under a multitude of different conditions. As the placenta is the least understood yet one of the most vital organs, not only for the viability of the pregnancy but also for the health of the offspring in years to come (Guttmacher, Maddox, & Spong, 2014), new reliable models for testing are desperately needed. As gestation and placental function and anatomy vary vastly among different species, models that come as close as possible to human in *vivo* physiology are necessary in order to make specific and sensitive predictions. Many studies of placental physiology and pathology have focused on animal models and

placental *ex vivo* explants. Although studies on these models are valuable to acquire new knowledge, the question remains whether they can provide information physiologically relevant to human *in vivo* placental physiology.

1.2.5.2 Animal models

Animal models have been proven not to be ideal for the study of human placental physiology, as many aspects, such as the high level of invasiveness of the trophoblast, are unique to humans (Carter, 2007; Orendi et al., 2011). Although studies on placental explants from humans benefit from investigating the exact tissues and cells needed to understand human placental physiology, these term placentas have often already reached the maximum stage of development, or have even begun to degenerate, possibly not accurately mimicking physiology in earlier stages of development, which are often crucial in development of gestational pathologies or fetal development. It is evident that the best models today for human organ physiology in cell culture, especially in the fields of cancer and toxicology, use 3-dimensional (3D) conditions, as extracellular parameters are more physiologically relevant, allowing cells to grow into organoid structures (Lelièvre, Kwok, & Chittiboyina, 2017). Some studies have shown that growing placental cells in 3D as opposed to 2D monolayers leads to a change in cell structure and physiology (James, Carter, & Chamley, 2012; McConkey et al., 2016; Muoth et al., 2016). Most of these studies, however, express a need for further research into 3D placental models to validate findings, as there is still a paucity of information regarding placental cell physiology within 3D conditions.

1.2.5.3 Villous explants

Different types of *ex vivo* and in *vitro* models for the study of placental physiology exist. To study actual placental tissue, studies have used placental (villous) explants. Most commonly, villous explants are used to describe effects on placental differentiation, proliferation, syncytial fusion and apoptosis (Orendi et al., 2011). The availability of fresh placental tissue is hard to come by, and experiments must be done immediately after placental delivery. Another method of human placental testing is using primary trophoblasts in culture. Placental tissue is trypsinized and various other steps which have been validated over the years are used to purify villous trophoblasts to use in cell

culture. Studies have used early placental cells from chorionic villus sampling (Seeho, Park, Rowe, Morris, & Gallery, 2008), but most studies use term placental tissue to extract primary trophoblasts (Orendi et al., 2011; Seeho et al., 2008).

1.2.5.4 Placental cell lines

Although the study of primary trophoblasts is an ideal model when considering that cells are directly harvested from human placentae and do not need to be immediately used as they can be cryopreserved, a substantial disadvantage to primary trophoblasts is their inability to proliferate in culture, resulting in a culture time of only a couple of days, making long-term experiments unfeasible (Orendi et al., 2011). In order to circumvent small culture windows, most studies employ trophoblastic cell lines for cell culture, which have been immortalized and can be passaged multiple times. Cell lines that have been derived from primary trophoblasts and transfected with the SV40 virus such as the SGHPL group and the HTR-8/SVneo cell line benefit from their primary trophoblast origin, however, these cells are HLA and sometimes vimentin positive, whereas the primary trophoblast does not express HLA or vimentin and therefore should not be considered when searching for a surrogate (King, Thomas, & Bischof, 2000; Orendi et al., 2011). Cell lines derived from choriocarcinoma, such as BeWo, JEG-3 and Jar, although not derived from primary trophoblasts, show similar characteristics of primary trophoblasts, such as the expression of hCG, hpl, estradiol, estrone and progesterone as well as being vimentin and HLA negative in the case of BeWo and JEG-3 cell lines (Wolfe, 2006). Additionally, BeWo cells retain the ability to form syncytia when being treated with forskolin or 8-Br-cAMP (Orendi et al., 2011; Wice, Menton, Geuze, & Schwartz, 1990; Wolfe, 2006), one of the reasons this cell line is highly valued in the study of placental physiology (Wolfe, 2006). In this study, two different cell lines derived from choriocarcinoma, BeWo and JEG-3 are used to model the human placenta.

1.3 Aims and objectives

1.3.1 General hypothesis

To date, there is a paucity of research focusing on the effects of BPA on the human placenta. As placental function is crucial to fetal development and pregnancy outcome, the general aim of this project is to elucidate how BPA affects placental function. As there is need for relevant placental models that accurately represent the human placenta, we have developed a 3D placental model using the BeWo placental cell line.

1.3.2 Aims

1. To validate the cell-lines BeWo and JEG-3 as *in vitro* placental models by determining expression of estrogen receptors and validate the syncytialisation process of the BeWo cell line.

2. To determine the effects of BPA on the placental cell lines BeWo and JEG-3, by studying effects on: cell proliferation, regulation of ERs, and changes in the phosphorylation status of key MAPK.

3. To establish a comprehensive map of genes that are altered in vitro, following treatment with BPA using a non-biased screen (microarray analysis).

4. To validate a 3D placental model using BeWo cells and placental explants.

5. To compare results with term placental villous explants that have been treated with BPA for receptor regulation, gene upregulation, hormone expression and syncytialisation.

Chapter 2

Methodology

2.1 Tissue Culture

2.1.1 Cell Lines

BeWo**:**

Choriocarcinoma derived cell line with human trophoblastic qualities able to cellularly differentiate in vitro to syncyciotrophoblast cells using 8-bromo-cAMP or forskolin (Kristina Orendi, Gauster, Moser, Meiri, & Huppertz, 2010; Pattillo & Gey, 1968; Wice et al., 1990).

Karyotype	Cellular product
Modal number =86; range=71 to 78 Stemline number is hypotetraploid	hormones: progesterone; human chorionic gonadotropin (hCG); human chorionic sommatotropin (placental lactogen); estrogen; estrone; estriol; estradiol. Keratin.

Figure 2.1 Characteristics of the BeWo cell line (ATCC® CCL-98™), adapted from www.lgcstandards-atcc.org, copyright 2016.

JEG-3:

Human placental choriocarcinoma cell line derived from serial cloning of BeWo (Pattillo & Gey, 1968).

Karyotype	Cellular product
Hypertriploid. Modal number =71	Human chorionic gonadotropin (hCG), human chorionic somatomammotropin (human placental lactogen), progesterone

Figure 2.2 Characteristics of the JEG-3 cell line (ATCC® HTB-36™), adapted from www.lgcstandards-atcc.org, copyright 2016.

2.1.2 Tissue culture practice

Maintenance of an aseptic environment was assured by using a HERAsafe laminar flow cabinet (Heraeus). Equipment and surfaces were disinfected with continued applications of 70% industrial methylated spirits (IMS) dissolved in dH_2O. Sterilised flasks and plasticware were used or sterilized by autoclaving, all non-sterile.

2.1.3 Maintenance of cell lines

BeWo cells were grown in Ham F12 Medium (Gibco™) supplemented with 10% fetal bovine serum (FBS) (Gibco™) and 0.1% of Penicillin/Streptomycin to avoid cross-contamination. The cells were maintained in 75cm^2 non-treated culture flasks (Nunc™) under standard culture conditions in a humidified atmosphere containing 5% CO_2 at 37°C. The cells were confluent after 48 to 72 hours and subculturing of BeWo cells consisted in splitting the cells 1:3 or 1:5 into 19ml of complete Ham F12 medium per culture flask. The cells were detached by incubating in 2.5 ml of TrypLE™ Express (Gibco™) for a few minutes and by tapping the culture flask gently. Fresh media was added and cells were split 1:3 or 1:5. Media was changed every 24-48 hours according to supplier's suggestions to prevent glucose exhaustion in the culture.

JEG-3 cells were maintained in Minimum Essential Medium (MEM) (1X) + GlutaMAX (Gibco™) supplemented with 0.1% Penicillin/Streptomycin to avoid cross-contamination (Gibco™) and 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco™). The same procedure for subculturing BeWo cells was used for JEG-3 cells and cells were kept in standard culture conditions.

2.1.4 Seeding of JEG-3 cell line and BeWo cell line for treatment

Cells were plated on 6-well plates (Nunc™) at a specific seeding density with 2 ml of the appropriate media. Cells were grown and allowed to attach for 24 hours and incubated at standard culture conditions. After 24 hours, cells were treated depending on the experiment.

2.1.5 Thawing cells

Before removing frozen cells from liquid nitrogen, a 75^2 cm flask containing 19ml of fresh media was incubated under standard culture conditions for 2 hours, for the media to equilibrate. Next, a cryovial (Nalgene) of frozen cells was removed from liquid nitrogen and thawed carefully in the water bath at 37°C. When the cell suspension was completely defrosted, the solution was transferred into the flask with a pipette and then moved into the incubator overnight to allow the cells to adhere. The next morning, media was replaced with fresh media in order to remove the excess of DMSO that was used to freeze the cells down.

2.1.6 Cryopreserving cells

In order to make stocks of cells, several flasks of cells were grown under standard culture conditions. First, the media was aspirated and the cells were incubated with 2.5ml TrypLE™ Express for a few minutes in order for the cells to detach from the flask surface. Cells were diluted in 5ml of media and then centrifuged at 12,000rpm for 5 minutes. Supernatant was discarded and the cell pellet was resuspended in cold, fresh media containing 10% FBS, 1% penicillin/streptomycin and 10% dimethyl sulfoxise (DMSO) (Fisher Bioreagents™). 1 ml of this solution was pipetted into each cryovial.

The cryovials were frozen gradually in a container (Nalgene), prior to being moved to liquid nitrogen after 24 hours.

2.2 Syncytialisation of BeWo cell line

2.2.1 Syncytialisation using forskolin

BeWo cells grown in 75cm^2 flasks were seeded onto 6 well plates and left to grow in the incubator at standard culture conditions with 2 ml of media. Forskolin (Fisher Bioreagents™) was dissolved in DMSO at a concentration of 0.1M. BeWo cells were treated with 50µM and 100µM forskolin over a total period of 72 hours. Forskolin treatment was renewed every 24 hours.

2.2.2 Syncytialisation using 8-Bromo-cAMP

BeWo cells were seeded onto 6 well plates and left to grow for 24 hours in an incubator at standard culture conditions with 2 ml of media. Media was changed and cells were treated with 50µM 8-Bromo-cAMP (Tocris Bioscience™) dissolved in sterile H_2O for 72 hours.

2.3 Treatment of BeWo Cell line with Bisphenol A (BPA)

Cells were plated on 6-well plates at a specific seeding density with 2 ml of the appropriate media. Cells were grown for 24 hours and incubated at standard culture conditions. After 24 hours, media was changed and cells were treated for 24 hours with 3 nM Bisphenol A (BPA) (Sigma-Aldrich®) dissolved in ethanol, 10 nM BPA, 30 nM β-estradiol (E2), or pure ethanol as a control. After 24 hours, cells were processed for further experiments.

2.4 3D Cell Culture

2.4.1 3D cell culture using Matrigel®

A Millicell 8-well EZslide was used to grow cells in 3D. Matrigel Matrix (Corning) was thawed and kept cold. 50 µl Matrigel® was pipetted into each well avoiding bubbles and the slide was placed in the incubator at 5% CO_2 and 37°C for 15 minutes to solidify. In the meantime, BeWo cells grown in 75cm^2 flasks were grown in 3D. First, media from flasks was removed and cells were incubated for a couple of minutes with 2.5 ml of TrypLE. The flask was given a tap to dislodge cells and 5ml of media were added to the flask. After some resuspension, cells were transferred to a 15ml tube. Cells were counted using FastRead 102 disposable counting chambers (Immune Systems). Cells were diluted with media to yield 10,000, 15,0000, 20,000 and 25,000 cells/ml depending on experiment. Equation used:

Each counting chamber consists of ten 4x4 grids. The volume above each 4x4 grid is 10^{-4}ml (0.1ml). The concentration (counts/ml) is given by:

$$\text{counts/ml} = \frac{\text{total counts}}{\text{number of complete 4x4 grids counted}} \times 10^4 \times \text{sample dilution (if any)}$$

1800 µl of media containing 2% Matrigel® was prepared, and 1800 µl diluted single cell suspension was added to this. 400 µl of media plus Matrigel® was added to each well, and slides were left in an incubator at 5% CO_2 and 37°. Media was changed adding 400 µl of media with 2% Matrigel® to each well every four days.

2.4.2 Concentration optimisation of BeWo 3D culture

Concentration of seeded BeWo cells/ml was optimised for 3D cell culture by seeding four different concentrations on one slide and imaging cell growth every day. Wells 1 and 2 were seeded at a density of 5,000 cells/ml, wells 2 and 3 were seeded at a

density of 7,500 cells/ml, wells 4 and 5 were seeded at a density of 10,000 cells/ml and wells 7 and 8 were seeded at a density of 12,500 cells/ml. Optimal seeding density was determined by confluence of cells after 12 days of growth.

2.4.3 Time point optimisation of BeWo 3D culture

Optimal growth time of BeWo cells in 3D was determined by seeding cells at 10,000 cells/ml at different time points. Wells 1 and 2 were seeded at day zero, wells 3 and 4 were seeded at day four, wells 5 and 6 were seeded at day eight, and wells 7 and 8 were seeded at day 12. Optimal growth time of BeWo cells was determined by confluence of cells after 4, 8, 12, and 16 days.

2.4.4 3D cell culture using GrowDex® hydrogel

Cells were first grown in 75cm² flasks until confluent (Nunc™). Growdex® hydrogel was diluted with the appropriate media. 100 µl of hydrogel was added to each well of a Millicell® 8-well EZslide (Merck Millipore). Cells were incubated for a couple of minutes with 2.5 ml of TrypLE Express™. The flask was given a tap to dislodge the cells and 5 ml of media were added to the flask. After some resuspension, cells were counted using the FastRead 102 disposable counting chamber system as previously described. Cells were diluted to the appropriate concentration and 200 µl of cell suspension were added to the top of the hydrogel. After 7 days, 100µl was added and 100µl removed as to not disrupt the cells and this process was repeated every 7 days.

2.4.5 Concentration and time point optimization of 3D cell culture

Cells were seeded in concentrations of 40,000, 60,000 and 80,000 cells/ml and imaged every 5-7 days in order to determine optimal seeding density. Different concentrations of hydrogel were used: 0.5%, 0.8% and 1%. Cells were grown for 21 days. Imaging was used to determine the optimal time points of growth, optimal seeding density and optimal hydrogel concentrations.

2.5 Total RNA extraction from cells

2.5.1 Sigma-Aldrich GenElute Mammalian Total miniprep kit

Cells were grown in 6-well plates and treated as mentioned above. RNA was extracted using the GenElute Mammalian Total RNA miniprep kit (Sigma-Aldrich), following the manufacturer's instructions. Cells were lysed directly in the wells using Lysis solution complemented with an appropriate amount of β-mercaptoethanol (2-ME) per RNA preparation (10µl of 2-ME for each 1ml). The side of the wells was tapped so that the solution would cover the surface of the well. The culture vessel was let to sit for approximately 2 minutes and then tilted on the side to allow collection of the cell lysate. To allow the removal of cellular debris and DNA, the lysed cells were pipetted into a filtration column and then centrifuged at 13,000 x g for 2 minutes. The filtration column was discarded and an equal amount of 70% ethanol solution was added to the filtered lysate. After a brief vortex, approximately 700µl of solution was pipetted into the binding column and centrifuged at maximum speed for 15 seconds. The flow-through was discarded and this step was repeated. The column was then washed and centrifuged at maximum speed for 15 seconds. The flow-through was again discarded and the column was washed again with a wash solution diluted with ethanol. The column was centrifuged again at maximum speed for 15 seconds. The above step was repeated though this time the column was centrifuged at maximum speed for 2 minutes to dry the binding column. 50µl of elution solution was added to the column, which was centrifuged for 1 minute. RNA was stored at -70°C.

2.5.2 Qiagen RNeasy Mini Kit

Cells were grown in 6-well plates and treated as mentioned above. RNA was extracted using the Qiagen RNeasy Mini Kit following the manufacturer's instructions. Media from cells was removed and wells were washed with PBS. 500 µl of Lysis solution containing 5 µl ß-mercaptoethanol was added to each well. The wells were tapped to dislodge cells and left for 2 minutes. The vessel was tilted to the side and the cell lysate was collected into one tube per well. Tubes were vortexed for homogenization. One volume of 70% ethanol was added to each tube. Up to 700 µl of each sample

were transferred to an RNeasy spin column and placed in a collection tube. The lysate was centrifuged at 8000 x g for 15 s. The flow through was discarded. The collection tube was reused and 700 µl buffer RW1 was added to the spin column. The spin column was centrifuged at 8000 x g for 15 s and the flow-through was discarded. The collection tube was reused and 500 µl buffer RPE was added to the spin column. The spin column was centrifuged at 8000 x g and the flow-through discarded. This step was repeated and the spin column was centrifuged for two minutes. The spin column was placed in a new 2 ml collection tube and centrifuged at full speed for 1 min. The spin column was placed into a new 1.5 ml collection tube and 40 µl RNAse-free water was added to the membrane. The column was centrifuged for 1 min at 8000 x g. RNA was stored at -70°C.

2.5.3 RNA Quantification

Every sample was measured using the NanoDrop 2000C (Thermo Fisher Scientific) spectrophotometer. Concentration was calculated at 280 nM and purity was assessed by A_{260}/A_{280} ratio, a range of 1.7-2.0 classified as acceptable.

2.6 cDNA synthesis

2.6.1 Precision NanoScript™ 2 Reverse Transcription Kit

cDNA synthesis was performed using the Precision NanoScript™ 2 Reverse Transcription Kit (Primerdesign). Following the manufacturer's protocol, RNA samples were diluted in water to normalise the concentration across all samples and reach a final volume of 9µl and incubated with 1µl of random nonamer primer at 65°C for 5 minutes. Samples were transferred directly from 65°C to ice. 10µl of mastermix (nanoScript2 4x buffer, dNTP mix 10mM, RNAse free water and nanoScript2 enzyme) was added to the existing 10µl of the samples on ice. Samples were briefly vortexed, spun and incubated at 25°C for 5 minutes and then at 42°C for 20 minutes. The reaction was heat inactivated by incubating at 75°C for 10 minutes. cDNA samples were stored at -20°C until use.

Components	1 Reaction
RNA template (up to 2)µL	x µL
RT primer (yellow) and/or (red) or (green)	1.0 µL
RNAse/DNAse free water (white)	x µL
Final volume	10 µL

Figure 2.3 Components in one reaction for cDNA synthesis composed of the RNA template, random primers and nuclease free water (adapted from Precision NanoScript™ 2 Reverse Transcription Kit handbook, version HB05.5.03, published 29.01.2016).

2.6.2 High Capacity cDNA Reverse Transcription Kit

cDNA synthesis was performed using the High Capacity Reverse Transcription Kit (Applied Biosystems). First, the 2X-RT mastermix for a 20µl reaction was prepared.

Component	Volume/Reaction (µL)	
	Kit with RNase Inhibitor	Kit without RNase Inhibitor
10X RT Buffer	2.0	2.0
25X dNTP Mix (100 mM)	0.8	0.8
10X RT Random Primers	2.0	2.0
MultiScribe™ Reverse Transcriptase	1.0	1.0
RNase Inhibitor	1.0	—
Nuclease-free H_2O	3.2	4.2
Total per Reaction	10.0	10.0

Figure 2.4 Components in the 2X-RT mastermix for each reaction for a 20µl reaction when using the High Capacity cDNA Reverse Transcription Kit (adapted from the High Capacity cDNA Reverse Transcription Kit handbook, ©2006,2007,2010).

10μl of mastermix was pipetted into a 0.2 μl tube for each sample and kept on ice. 10 μl of RNA sample was pipetted into each tube and tubes were briefly centrifuged. Tubes were placed into the thermal cycler at 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 minutes. cDNA samples were stored at -20°C until use.

2.7 Real Time Polymerase chain reaction (qPCR)

2.7.1 PrecisionPlus mastermix and Quantstudio™ 7 Flex Real-Time PCR System

PrecisionPlus mastermix premixed with SYBR green (Primerdesign) was used for qPCR. A QuantStudio™ 7 Flex System Real-Time PCR System machine (Applied Biosystems) was used for the experiment. Samples were loaded on a 96-well plate containing cDNA (5μl), mastermix, water and primers specific for each gene, following the manufacturer's instructions.

Components	1 Reaction
Precision™ 2X qPCR Mastermix premixed with SYBRgreen	10 μL
Primers (6pmols Forward and Reverse)	x μL
Template (25ng)	x μL
RNAse/DNAse free water (up to final volume)	x μL
Final volume	20 μL

Figure 2.5 Components of each reaction in a 20μl reaction using PrecisionPlus mastermix (Primerdesign) (adapted from the Precision™ 2X qPCR Mastermix handbook).

A relative quantification was performed and all values were normalised to the housekeeping gene. The relative quantification produces an amplification curve which shows the number of cycles versus the intensity of fluorescence. As the cycles

increase, more double-stranded DNA is produced and binds to SYBR green, which is then detected by the machine. The cycle in which this happens is called the 'cycle threshold' (Ct). In order to distinguish between specific binding and unspecific binding to other DNA products or primer dimer complexes, a dissociation curve is acquired. This shows the melting curve of the primer products which are specific and previously known and other, if any, by-products. So to evaluate the quality of the results both curves need to be obtained and checked. The control RQ value is set to 1.

For cell culture samples the following equation was used:

$$\Delta Ct = Ct \text{ (gene of interest)} - Ct \text{ (housekeeping gene)}$$

$$\text{Relative Quantity (RQ)} = 2^{-\Delta Ct}$$

This equation quantifies relative fold change in gene expression when comparing the gene of interest to the housekeeping gene.

2.7.2 Primers

Primer sequences were taken from various scientific publications found by searching for primer sequences at www.ncbi.nlm.gov. All primers were created with the Sigma-Aldrich® custom DNA oligo synthesis programme and showed no evidence of dimerization.

Gene	Sequence	Source
CPEB1	**F:** 5'TTTCAAGCCTTCGCATTTCCC3'	(Galardi et al., 2016)
	R: 5'GGACCCAACGCCATCTTTA3'	
CAV1	**F:** 5'ACCCACTCTTTGAAGCTGTTG3'	(Deb et al., 2014)
	R: 5'GAACTTGAAATTGGCACCAGG3'	
	R: 5'TGCACTCTGACCATGAACCA3'	
ITGA1	**F:** 5'CAGCAAGAAAGGAGGCATTC3'	(Xiaoqin Liu et al., 2017)
	R: 5'TTTCCTCGGTTATAGCTGCC3'	
Leptin	**F:** 5'CCTGACTGGTGCTATAGGCTGGA3'	(Y. Pan et al., 2017)
	R: 5'GTGAGTGCGGTTTGACCACTG3'	
hPLAC8	**F:** 5'GGGTGTCAAGTTGCAGCTGAT3'	(Kaistha et al., 2016)
	R: 5'TAGATCCAGGGATGCCATATCG3'	
GABBR	**F:** 5'GGAAGAGGTCACCATGCAG3'	(Plummer et al., 2011)
	R: 5'AGTTTCCCAGGTTGAGGATG3'	
ERα	**F:** 5'GCCCTCCCTCCCTGAAC3'	(Silva et al., 2010)
	R: 5'TCAACTACCATTTACCCTCATC3'	
ERβ	**F:** 5'TCCTCCCAGCAGCAATCC3'	(Silva et al., 2010)
	R: 5'CCAGCAGCAGGTCATACAC3'	
GPR30	**F:** 5'GTTCCTCTCGTGCCTCTAC3'	(Silva et al., 2010)
	R: 5'ACCGCCAGGTTGATGAAG3'	
TOP1	**F:** 5'CCTTCCCTCTCTCCCATTTC3'	https://www.ncbi.nlm.nih.gov/tools/primer-blast/
	R: 5'AGCCACGACTGCTTCAAGTT3'	

Figure 2.6 Primer sequences of all primers used for qPCR experiments and the sources used to create the primers. Sequences were either taken from published papers or created using the primer-blast tool on ncbi.nlm.nih.gov.

2.7.3 geNORM™ analysis for qPCR:

Ideal housekeeping gene for qPCR analysis was established using the geNORM™ kit and qBase+ software (Biogazelle). Data from qPCR was uploaded to the software and values for expressional stability were calculated to determine the optimum reference gene. Six housekeeping genes were used using the 6-gene geNORM™ kit (ACTB, SDHA, RPL13A, UBC, TOP1, GAPDH). 96-well plates were set up for qPCR as previously described using 2x PrecisionPLUS™ qPCR Mastermix, using all cDNA samples and all 6 housekeeping genes. After this, targets were defined within the qBase+ software and data from the qPCR machine was uploaded to the software. Then, data was analysed to produce an "M" value for each gene which defines the expression stability of the gene within the sample, and shows which gene is most stably expressed among all samples.

2.8 Immunofluorescence

2.8.1 Immunoflourescence of cells grown in monolayer

Cells were grown and seeded at a specific density in a 6-well plate that contained coverslips. Cells were allowed to adhere for 24 hours and then fixed with 4% PFA (Sigma-Aldrich) for 10 minutes at room temperature. Cells were then permeabilised with 0.5% Triton X-100 (Sigma- Aldrich) on ice for 5 minutes. Cells were blocked in 5% FBS-PBS (v/v) for 45 minutes and then incubated for 45 minutes with monoclonal antibodies or which were diluted in 5% FBS-PBS (v/v). Cells were washed and then incubated with a staining buffer that contained Alexafluor 568 secondary anti-rabbit or anti-mouse antibody (1:1000) and phalloidin 488 (50μg/ml) (Sigma-Aldrich®) (to stain f-actin, cytoskeleton) depending on the experiment. This incubation was done in the dark for 45 minutes. After another wash with 5% FBS-PBS (v/v), the coverslips were mounted on slides with Vectashield Hard Set™ Mounting Medium with DAPI (Vector laboratories) and visualised on a HF14 Leica DM4000 microscope.

2.8.2 Immunofluorescence of phospho-targets of cells grown in monolayer

BeWo cells were grown and seeded at a specific density in a 6-well plate that contained coverslips as above. After 24 hours, media was changed to 0.5% FBS starved media and left to incubate for an hour. Then, cells were treated with BPA (3 nM, 10 nM) and E2 (30 nM) for 24 hr, 1 hr, 30 min, 15 min and 5 min. Cells were fixed with cold 4% PFA as mentioned above. Cells were stained as previously mentioned using appropriate antibodies.

Antibody	Company	Dilution
ER-α (F-10) SC8002	Santa Cruz® Biotechnology	IF: 1:100
ER-β (B-3) SC373853	Santa Cruz® Biotechnology	IF: 1:100
GPR30 (N-15)-R SC48525-R	Santa Cruz® Biotechnology	IF: 1:100
Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb #4060	Cell Signaling Technology®	WB: 1:2000 IF: 1:400
Akt (pan) (11E7) Rabbit mAb #4685	Cell Signaling Technology®	WB: 1:1000 IF: 1:100
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody #9101	Cell Signaling Technology®	WB: 1:1000 IF: 1:250
P44/42 MAPK (Erk1/2) (137F5) Rabbit mAb #4695	Cell Signaling Technology®	WB: 1:1000 IF: 1:100
p38 MAPK Antibody #9212	Cell Signaling Technology®	WB: 1:1000 IF: 1:50
Phospho-p38 MAPK (Thr180/Tyr182) Antibody #9211	Cell Signaling Technology®	WB: 1:1000 IF: 1:800
E-Cadherin (4A2) Mouse mAb #14472	Cell Signaling Technology®	WB: 1:1000 IF: 1:50
E-Cadherin (4A2) Mouse mAb (Alexa Fluor® 488 Conjugate) #86770	Cell Signaling Technology®	IF: 1:50
Pan-Keratin (C11) Mouse mAb #4545	Cell Signaling Technology®	WB: 1:1000 IF: 1:400
Anti-mouse IgG, HRP-linked Antibody #7076	Cell Signaling Technology®	WB: 1:1000
Anti-rabbit IgG, HRP-linked Antibody #7074	Cell Signaling Technology®	WB: 1:1000
Anti-mouse IgG (H+L) $F(ab')_2$ Fragment (Alexa Flour®647 Conjugate) #4410	Cell Signaling Technology®	IF: 1:1000
GAPDH (D4C6R) Mouse mAb #97166	Cell Signaling Technology®	WB: 1:1000
Ob Antibody (A-20): sc-842	Santa Cruz® Biotechnology	IF: 1:100
Caveolin-1 Antibody (7C8) sc-53565 Alexa Fluor® 488	Santa Cruz® Biotechnology	IF: 1:100
Anti-SIM2 Antibody (HPA029295)	Atlas Antibodies	IF: 1:100
CLASP2 Antibody (F-3) sc-376496	Santa Cruz® Biotechnology	IF: 1:100

Figure 2.7 Antibodies used in all immunofluorescence staining and Western blots including dilutions. IF: Immunofluorescence. WB: Western blot.

2.8.3 Immunofluorescence of BeWo cells grown in 3D

2.8.3.1 Cells in Matrigel® (Corning®)

Media was aspirated from each well and cells were fixed with 400µl of 2% PFA in 1% PBS (1:1 dilution) for 20 minutes at room temperature on a rocker. To permeabilize, 200 µl/well of PBS containing 0.5% Triton was added for 10 minutes at 4°C. Each well was rinsed 3 times with 200µl PBS + 10% glycine solution and left for 10 minutes on the rocker for each rinse. To block, cells were incubated with 200µl/well IF buffer + 10% FBS for 1 hour at room temperature on the rocker. For secondary block, primary block was aspirated and 100 µl/well of secondary block solution was added and left for 30 minutes at room temperature on the rocker. The secondary block was aspirated and 100 µl/well of secondary block + primary antibody (1:100) was added and left overnight at 4°C. The primary antibody was aspirated and the wells were rinsed 3 times with IF buffer and left on the rocker for 20 minutes each time. 100 µl/well of secondary antibody (1:200) + IF buffer + 10% FBS was added to each well and left for 40 minutes on the rocker. Wells were rinsed 3 times with 200 µl/well IF buffer and left on the rocker for 20 minutes each time. Cells were mounted with Vectashield Hard Set™ Mounting Medium with DAPI (Vector laboratories) using the coverslip and removing bubbles. Slide was left to dry overnight at room temperature in the dark.

2.8.3.2 Membrane Staining of 3D BeWos in GrowDex®

Cell membranes of BeWo cells seeded onto GrowDex® (UPM Biochemicals) were stained using the PKH7 Fluorescent Cell Labelling Mini Kit (Sigma-Aldrich®). A suspension containing $2x10^7$ cells/ml in a conical tube was washed with serum-free medium. This was then centrifuged at 400 *x g* for 5 minutes. The supernatant was aspirated and a 2x cell suspension was prepared by adding 1ml of diluent C to the pellet and resuspending. 4 µl of PKH7 ethanolic dye solution were added to 1ml of diluent C in a separate tube. The cell suspension with dye was added to the 1ml of 2X dye solution and mixed by pipetting. The solution was incubated for 1-5 minutes with periodic mixing. The staining was stopped by adding an equal volume (2ml) of FBS and incubating for 1 minute. The mixture was centrifuged at 400 *x g* for 10 minutes at

20-25˚C. the supernatant was removed and the pellet resuspended in a fresh tube with 10 ml of complete medium. The suspension was centrifuged for 5 min at 400 *x g* at 20-25˚C. The suspension was washed 2 more times with 10ml of complete medium and resuspended in complete medium and used to set up 3D cell cultures using GrowDex® (UPM Biochemicals) as described above.

2.9 ImageStream

Protein expression and localization were investigated using ImageStream® MKII (Amnis®) high resolution flow cytometry.

2.9.1 Fixing Cells

Expression and cellular location of ERα and ERß was assessed using ImageStream® MKII (Amnis®) imaging flow cytometry. Cells were cultured in T-75 tissue culture flasks (Nunc™) until 80-90% confluent. Media was aspirated and the cells were incubated with 2.5mL of TrypLE Express (Invitrogen) per ~75c^2 growth surface area and the flask was manually disturbed to detach adherent cells which were resuspended in the appropriate media. Cells were then transferred to a 15 ml tube and centrifuged for 5 minutes at 1200 RPM. The supernatant was removed and the cells were resuspended in ice cold 4% PFA (Sigma-Aldrich®) for 5 minutes to crosslink cellular proteins. The cell suspension was centrifuged for 2 minutes at 1200 RPM and the PFA removed. The cells were permeabilized with 0.5% Triton™ X100 (Sigma-Aldrich®) in PBS on ice for 5-10 minutes. The cells were transferred equally to eppendorf tubes and centrifuged in a microcentrifuge.

2.9.2 Staining Cells

The cells were incubated in blocking buffer (5% bovine serum in PBS) for 30 minutes with gentle agitation. Cells were centrifuged for 3 minutes at 2000 RPM and the blocking buffer was removed. The cells were then incubated in the appropriate primary antibody (ERα, ERβ, GPR30, Cell Signaling Technology®) diluted 1:100 in blocking buffer overnight at 4°C with gentle agitation. Following primary antibody incubation,

cells were centrifuged for 3 minutes at 2000 RPM and the antibody removed. The cells were resuspended in PBS to remove any remaining antibody and centrifuged again for 3 minutes at 2000 RPM. From this step onwards the cell were protected from light as the fluorophore conjugated to the secondary antibody is light sensitive. The PBS was removed and the cells incubated in secondary antibody (Alexa Flour 568 secondary anti-mouse antibody, 1:1000, Cell Signaling Technology®) diluted in blocking buffer for 1 hour with gentle agitation. After secondary antibody incubation the cells were centrifuged for 3 minutes at 2000 RPM and the secondary antibody removed. The cells were resuspended in PBS to remove any remaining antibody and centrifuged for 3 minutes at 2000 RPM. PBS was removed and the cells were resuspended in 30µL Accumax (Innovative Cell Technologies) to dissociate any cellular aggregates. 1µL of Draq5 nuclear stain (Thermo Fisher Scientific) was added before visualisation on ImageStream®.

2.10 Western Blot

2.10.1 Treatments

BeWo and JEG-3 cells were grown on 6-well plates for 24 hours, media was changed to starved media (0.05% FBS) and cells were treated with BPA (3 nM, 10 nM) and β-estradiol (E2, 30 nM) as a positive control for 5, 15, 30 and 60 minutes as well as 24 hours. Media was aspirated and cells were washed with PBS. PBS was aspirated and cells were kept cold and 200µl Laemmli buffer (Sigma-Aldrich®) was added. Lysates were homogenized using a cell scraper and transferred to eppendorf tubes. Samples were boiled for 5 minutes at 90˚C and stored at -80˚C.

2.10.2 Coomassie Staining

Gel electrophoresis was performed with samples by preparing a 10% resolving gel and a 5% stacking gel (see table and exact procedure below). Samples were loaded into the gels and run at 40mA per gel and 300V with a 10% SDS page running buffer (see table below). Gels were stained with coomassie blue staining buffer (0.1% Coomassie Brilliant Blue R-250, 50% methanol, 10% glacial acetic acid) for 1 hour at

room temperature. Gels were then destained with destainer (40% methanol, 10% glacial acetic acid) for 20 minutes 3-5 times and then stored in water at 4˚C. This was done to visualize evenness of band formation.

2.10.3 Western Blotting

For Western blots, 10% resolving gels and 5% stacking gels were prepared (see table below). The resolving gel was pipetted between two glass plates and 100% methanol was poured on top and the gel was left to set for 20 minutes. The methanol was removed and the stacking gel pipetted on top of the resolving gel and a comb was inserted to create wells for the sample. The gel was left to set for another 15 minutes.

Component	Amount
H_2O	7.9 ml
30% acrylamide mix	6.7 ml
1.5 M Tris (pH 8.8)	5.0 ml
10% SDS	0.2 ml
10% ammonium persulfate	0.2 ml
TEMED	0.008 ml

Figure 2.8 Components and amounts for preparing a 10% resolving gel (2 gels). Tris = tris(hydroxymethyl)aminomethane; SDS = sodium dodecyl sulfate; TEMED = Tetramethylethylenediamine.

Component	Amount
H_2O	2.7 ml
30% acrylamide mix	0.67 ml
1.5 M Tris (pH 8.8)	0.5 ml
10% SDS	0.04 ml
10% ammonium persulfate	0.04 ml
TEMED	0.004 ml

Figure 2.9 Components and amounts for preparing a 5% stacking gel (2 gels). Tris = tris(hydroxymethyl)aminomethane; SDS = sodium dodecyl sulfate; TEMED = Tetramethylethylenediamine.

The gels were loaded into a basin filled with 10% SDS-page running buffer (see table below). The samples and the ladder (PageRuler™ - Prestained Protein Ladder (Life Technologies)) were loaded into the gels (5-7µl) after warming to 90°C and centrifuging. Gel electrophoresis was run at 40mA per gel and at 300V with a 10% SDS-page running buffer for about 45 minutes until the proteins were separated and bands were seen near the bottom of the glass. To transfer gels onto the membrane, gels were stacked with filter paper, sponges and the nitrocellulose membrane (Thermo Scientific) in transfer buffer (see table below) and transferred at 300V and 400mA in transfer buffer on ice.

Component	**Amount**
Tris base	30g
glycine	144g
SDS	10g
H_2O	1000ml

Figure 2.10 Components to prepare 10X SDS page running buffer. 1X was prepared by diluting 100 ml of 10X running buffer with 900 ml of H_2O. Tris = tris(hydroxymethyl)aminomethane; SDS = sodium dodecyl sulfate.

Component	**Amount**
Tris base	30.3 g
glycine	144.1 g
H_2O	1000ml

Figure 2.11 Components to prepare 10X transfer buffer. Transfer buffer was diluted to 1x using 100 ml of the buffer, 800 ml of H_2O and 100 ml of cold methanol. Tris = tris(hydroxymethyl)aminomethane.

Component	Amount
100 mM Tris pH 8	5ml
Coumaric acid	22 μl
luminol	50 μl

Figure 2.12 Components to prepare “solution A” for developing Western blot films (amount per membrane). Tris = tris(hydroxymethyl)aminomethane.

Component	Amount
100 mM Tris pH 8	5ml
30% hydrogen peroxide	3μl

Figure 2.13 Components to prepare “solution B” for developing Western blot films (amount per membrane). Tris = tris(hydroxymethyl)aminomethane.

After transfer, membranes were cut and blocked in 5% milk powder in 1x TBS Tween for an hour. Membranes were treated with primary antibody in 5% BSA at a dilution of 1:1000 and incubated at 4˚C overnight. Membranes were washed 3 times for 15 minutes with 1x TBS Tween. Secondary antibody diluted in 5% BSA at a dilution of 1:2000 was added to membranes and membranes were incubated at room temperature for 1 hour. Membranes were washed 3 times for 15 minutes with 1x TBS Tween. After adding developer solutions (solution A and B, see table below) to the membranes in the dark for 2 minutes, membranes were developed on film in the dark using a developer (Curix60, AGFA).

2.11 Microarray

Two-colour microarray-based gene expression (low input Quick Amp labelling) was measured using Agilent Gene Expression oligo microarrays using a Sure Scan microarray scanner (Agilent). RNA was extracted from samples treated with 3 nM and 10 nM BPA as previously described. A Low Input Quick Amp Labelling Kit, Two-Colour (Agilent) was used to generate fluorescent cRNA using 100 ng of RNA as input. cRNA was quantified using a NanoDrop™ (ThermoScientific™) Spectrophotometer. For

Hybridisation, the hybridisation samples were prepared by adding the cRNA, 10x Gene Expression Blocking agent, nuclease free water and 25x Fragmentation buffer into a nuclease-free centrifuge tube. The samples were incubated at 60˚C for 30 minutes to fragment RNA and then cooled on ice for 1 minute. 2x hi-RPM Hybridisation Buffer was added to stop the fragmentation reaction. The samples were mixed and centrifuged at 13000 rpm and then loaded onto the array. The samples were hybridized at 65˚C for 17 hours. The microarray slides were washed twice with 100% isopropyl alcohol for 5 minutes and rinsed 5 times with Milli-Q water before washing with Agilent Gene Expression Wash buffer 1 and Gene Expression Wash buffer 2 according to the protocol. The slides were then put into slides holders in order to scan. Scanning and feature extraction were performed on a SureScan microarray scanner (Agilent) using feature extraction programme 12.0.

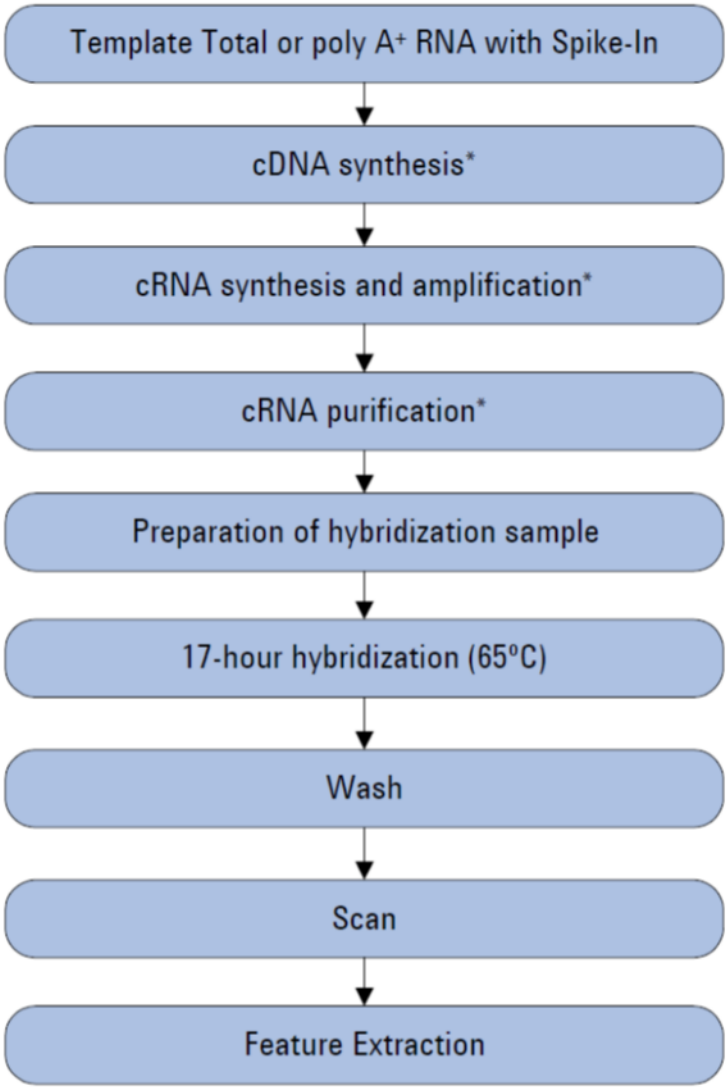

Figure 2.14 Overview of workflow for Agilent Technologies Microarray manufactured with Agilent SurePrint Technology (from Two-Color Micorarray-based Gene Expression Analysis Protocol, version 6.9.1, August 2015).

2.11.1 Bioinformatic analysis of microarray data

Results were organized by amount of fold change and significance ($P<0.05$) and top relevant differentially regulated genes were selected within each condition (3 nM BPA, 10 nM BPA, syncytialised 3 nM BPA and syncytialised 10 nM BPA in BeWo cells, as well as 3 nM BPA, 10 nM BPA and 30 nM E2 in placental explants). Results were analysed using FunRich (Functional Enrichment Analysis Tool) (www.funrich.org) bioinformatics software as well as Enrichr (Chen et al., 2013) in order to assess significant pathways upregulated genes were involved in and to assess overlap of

upregulated genes within different conditions. Upregulated genes were validated using qPCR analysis.

2.11.1.1 FunRich Functional Enrichment Analysis Tool

Official gene symbols of differentially regulated genes of interest were inserted into the FunRich input list. Enrichment analysis was performed by selecting different database options within FunRich. FunRich has the option of assessing cellular process, biological process, molecular function, protein domains, site of expression, biological pathways, transcription factors and clinical phenotypes. All options were selected in order to maximize output and understand associations between genes and processes. By selecting the “interaction network analysis” function, gene interactions were enriched using the FunRich database. Enrichment analysis was achieved by uploading selected database and analyzing against the FunRich database. By clicking on the “chart” icon and selecting the database to be analyzed, a column graph depicting cellular processes, organized by p-value is generated. This chart can be generated for the enrichment of molecular function, biological process, biological pathway, protein domain, site of expression, transcription factor and clinical phenotype as well by clicking on the respective tabs. These functions can be used to compare datasets with each other by clicking on the “compare” icon. Interaction networks between genes were generated by clicking on the “interaction” icon. Venn diagrams were generated using the Venn diagram function in FunRich, by uploading separate datasets which were then analyzed for overlapping genes. Datasets used were non-syncytialised 3 nM BPA, non-syncytialised 10 nM BPA, syncytialised 3 nM BPA, syncytialised 10 nM as well as 3 nM BPA, 10 nM BPA, and 30 nM E2 in placental explants.

2.11.1.2 Enrichr Analysis Tool

Official gene symbols of differentially regulated genes of interest were inserted into the input list. Enrichr uses databases such as NCI Nature and Go Molecular Function in order to assess gene enrichment in terms of molecular function, biological processes, biological pathways, transcription factors, diseases, and other gene enrichment groups. After inputting relevant genes, Enrichr creates groups according to different

databases as well as different processes/functions. Datasets used were as described above.

2.12 Placental explants from term human placentae

2.12.1 Term 2D placental explant culture

The study was performed under the supervision of Dr Manu Vatish at John Radcliffe Hospital, University of Oxford. Placentae were obtained from normal vaginal births at term at John Radcliffe Hospital Women's Centre, from patients who previously gave informed consent and experiments were approved by the Central Oxfordshire Research Ethics Committee as previously described (Motta-Mejia et al., 2017). The placenta was placed into Dulbecco's Phosphate Buffered Saline solution (DPBS) and used within an hour of birth. Using antiseptic methods, the amnion and any blood clots were removed inside a tissue culture hood. 6 different areas of placenta were removed by cutting with sterile scissors, keeping the pieces about 10mm in diameter. These pieces were placed into 6 different sterile conical 50ml tubes containing DPBS and washed by inverting the tubes. The DPBS was removed and fresh DPBS added and the pieces were washed once more. This process was repeated until the DPBS looked clear or about 5 times. The pieces were placed into 6 sterile petri dishes containing DPBS. Pieces were pulled apart inside the petri dish with fine sterile tweezers into equal pieces measuring about 2mm in diameter and making sure that all connective tissue was removed. One piece from each petri dish was placed into each Costar® Netwell™ (Corning) well containing 2.5 ml Human Large Vessel Endothelial Cell Growth Medium (Cellworks) that was preincubated for at least one hour at 8% oxygen and 5% CO2. Explants were left overnight and then treated for 24 hours with 3 nM BPA, 10 nM BPA and 30 nM E2 as previously described. Explants were kept in RNAlater® (Thermo Fisher Scientific) for RNA extraction at a later time. Media from day 0, day 2 and day 3 was kept for evaluation of cell viability and growth.

2.12.2 Term 3D placental explant culture

For 3D term placental explant culture, explants were obtained as above. 1% GrowDex® was made up with medium as mentioned previously. 50µl of GrowDex® was seeded into each of the wells in use on a 96-well plate and incubated at 37˚C and 5% CO2 for 30 min. Using sterile forceps, one 2mm biopsy was placed into each well and 50µl GrowDex® added on top. The plate was incubated for 30 min and 100 µl Human Large Vessel Endothelial Cell Growth Medium (Cellworks) was added on top. Media was replaced every 5 days without disturbing the GrowDex® and was kept for analysis. Explants were kept for 24 days. Explants were kept in formalin or RNAlater® for future immunohistochemical analysis or RNA extraction.

2.12.3 RNA extraction from term placental explants

Tissue disruption was achieved by adding 0.5 ml of lysis buffer (Qiagen) to eppendorf tubes after removing RNAlater®. A sterile metal bead was added to each tube and the tubes were inserted into a Tissue Lyser II (Qiagen). Tissue was disrupted for one minute. Samples were centrifuged and the supernatant used for RNA extraction. RNA extraction was achieved as mentioned previously using the RNeasy Mini Kit (Qiagen).

2.13 Hormone Analysis

The amount of secreted hCG or E2 was determined by the Department of Biochemistry (University Hospitals Coventry and Warwickshire NHS Trust) using the Elecsys® electrochemiluminescence immunoassay (ECLIA) and the fully automated modular analytics E170 testing system from Roche Diagnostics (Mannheim, Germany). Results were expressed as IU/ml.

2.14 Statistical Analysis

All data from experiments were analyzed using the f-test determining whether data is equal or unequal in variance. A student t-test or ANOVA test was performed on data of equal variance in order to compare data sets and determine significance between

them. In unequal variance data, the Mann-Whitney U test was used to determine significance. Values were siginifcant at $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

Chapter 3

Validation of a placental *in vitro* model in 2D

3.1 Introduction

In order to develop an *in vitro* model for placental testing, it is important to determine baseline parameters in models that already exist. As previously described, the best model for our studies in 2D are well-established cell lines derived from trophoblastic tumours, such as BeWo and JEG-3, which express same markers of differentiation and express the same hormones as primary trophoblasts (Orendi et al., 2011; Wolfe, 2006). With the placenta being a transient endocrine organ sensitive to a multitude of hormones and secreting hormones such as hCG, hPL, estradiol, estrone and progesterone, studies on hormone receptor levels in a 2D cell culture system are crucial, as hormone receptor levels are determining factors in the physiological outcome of hormonal exposure. With hormone receptors having the potential to be down or upregulated affecting hormone binding, and since different types of receptors for the same hormone can activate multiple signalling pathways (Jia et al., 2015), it is important to understand the expression profiles of estrogen receptors in the relevant tissue. As one of the main aims of this study focuses on determining the effects of BPA, a xenoestrogen, on the placenta; this chapter aims to measure the levels of estrogen receptors in our trophoblastic cell lines, as well as compare estrogen receptor level expression and proliferation level between syncytialised and non-syncytialised BeWo cells. Syncytialisation is a process of maturation and differentiation by which cytotrophoblasts transform into syncytiotrophoblasts, becoming amorphous, multinucleated, fused cells that lose the cell membrane, stop proliferating and become more endocrine active (Chang et al., 2014; Cross, Werb, & Fisher, 1994). Although both cell types are present in the placenta throughout most of the pregnancy, there are generally more syncytiotrophoblasts in the 2nd and 3rd trimesters (Lakshmi Devi & Raghupathy, 2013). In order to create a model which resembles the human placenta *in vivo*, we have syncytialised the BeWo cell line and validated this syncytialisation.

As mentioned previously, mapping estrogen receptor expression in placental cells is crucial for understanding the effects of xenoestrogens. In 1993, Krishnan et al found that BPA has estrogenic activity, as it binds to estrogen receptors and induces

progesterone receptors among other determinants in MCF-7 cells (Ben-Jonathan & Steinmetz, 1998; Krishnan, Stathis, Permuth, Tokes, & Feldman, 1993). Studies have shown that estrogens such as the most commonly found 17ß-estradiol can both up- and downregulate estrogen receptor α and β (Castles, Oesterreich, Hansen, & Fuqua, 1997; Cimarosti et al., 2006; Donaghue, Westley, & May, 1999; Nephew et al., 2000; Costa et al., 2014; Robertson, Farnell, Lindahl, & Ing, 2002) depending on the cell type and conditions. Estrogen-dependent regulation of estrogen receptors in the placenta is not a well-documented subject. One study suggests that high levels of ERα in placentae of patients with pre-eclampsia when compared to normal placentae could be a compensatory mechanism due to the fact that these patients exhibit significantly lower levels of estrogen in the placenta (Yin et al., 2013), suggesting a role of ERs in the development of the disease.

Furthermore, as there is a difference between dominant cell types when comparing 1st to 3rd trimester placenta, it is also necessary to elucidate how estrogen receptor profiles shift during placental development. In the placenta, levels of ER expression are not well documented, however a few studies have shown that ERα significantly dominates over ERβ in early developmental stages, with ERβ increasing in expression over time (Bukovsky et al., 2003; Schiessl et al., 2006; Yin et al., 2013), indicating a profile shift between non-syncytialised and syncytialised trophoblasts. The G-protein coupled estrogen receptor GPR30 (membrane bound ER) has also been demonstrated to be present in first-trimester and third-trimester placental cells (Owman et al., 1996) Taking the overall paucity of data with regards to placental estrogen receptor expression throughout different stages of placental development into account, the main aim of this chapter was to define estrogen receptor expression on the transcriptional and protein level in syncytialised and non-syncytialised placental cell lines. This chapter has therefore been structured into two main aims:

1. To assess ER levels in non-syncytialised BeWo cells in the context of gene expression as well as protein expression

2. To validate a method of syncytialisation of BeWo cells and assess gene expression and protein expression of ERs in syncytialised BeWo cells.

3.2 Results

3.2.1 Gene Expression of Estrogen Receptor Genes in BeWo and JEG-3 Cell Lines

In order to quantify ERα, ERß and GPR30 gene expression in BeWo and JEG-3 placental cell lines, qPCR was performed on these cell lines investigating gene expression of these receptors. After Ct values were obtained from the Quant Studio Flex qPCR machine, the equation (above) was used to calculate RQ values demonstrating relative fold change compared to the housekeeping gene (TOP1, validated by GeNorm, see materials and methods).

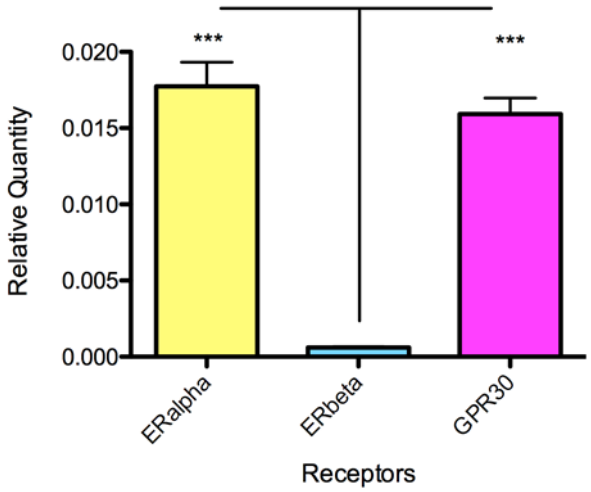

Figure 3.1 ERα is the predominant receptor in non-syncytialised BeWo cells. ERα and GPR30 genes are significantly more expressed than ERβ ($p<0.001$). Relative quantities (RQs) signify relative amount of gene expression when compared to the housekeeping gene, TOP1. RQ of ERα is 0.0175, RQ of GPR30 is 0.016, RQ of ERβ is approaching 0. Error bars depict standard error in the dataset.

In non-syncytialised BeWo cells, we have found that ERα and GPR30 genes are significantly more highly expressed ($p<0.001$) than gene expression of ERβ. The relative quantity of gene expression of ERα, meaning the expression when compared to the housekeeping gene, is 0.0175, whereas the relative gene expression of ERβ is approaching 0. Similarly, the relative gene expression of GPR30 is 0.016.

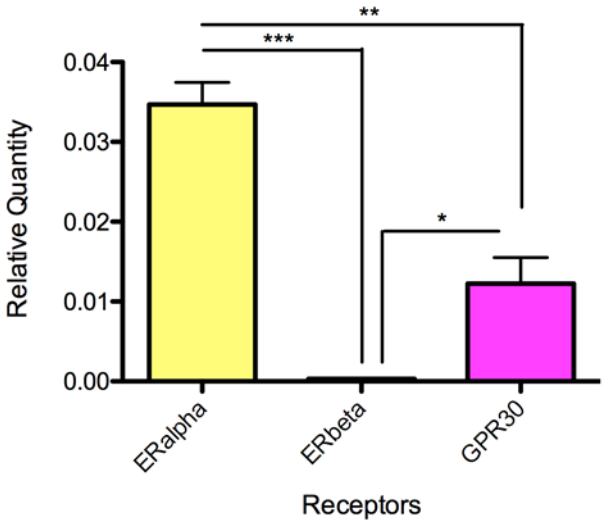

Figure 3.2 ERα gene expression is highest in JEG-3 cells. ERα expression is significantly higher than ERβ expression ($p<0.001$) and significantly higher than GPR30 expression ($p<0.01$). Similarly, GPR30 expression is significantly higher than ERβ expression ($p<0.05$). RQ of ERα is 0.035, RQ of GPR30 is 0.012, RQ of ERβ is approaching 0.

In JEG-3 cells, gene expression profiles were found to be similar to BeWo cells. ERα was the predominant receptor being significantly more expressed than ERβ ($p<0.001$) and GPR30 ($p<0.01$). GPR30 was the second-most highly expressed receptor and was significantly more highly expressed then ERβ ($p<0.05$). The relative quantity of ERα when compared to the housekeeping gene was 0.035, the relative quantity of GPR30 was 0.012 whereas the relative quantity of ERβ gene expression was approaching 0.

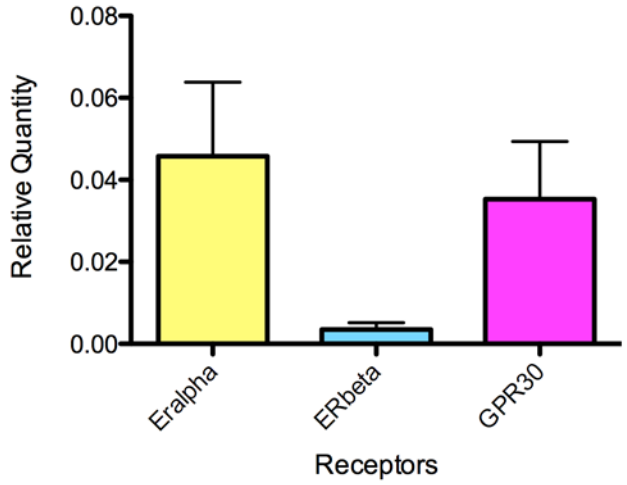

Figure 3.3 Estrogen receptor expression in syncytialised BeWo cells. ERα is the predominant receptor, followed by GPR30. There is no significant gene expression when comparing ERα or GPR30 to ERβ. RQ of ERα is 0.043, RQ of GPR30 is 0.038, RQ of ERβ is approaching 0.

In syncytialised BeWo cells, ERα was the most highly expressed estrogen receptor with an RQ value of 0.043, followed by GPR30 with an RQ value of 0.038. The RQ value of ERβ was approaching 0. There was no significant expression of any ER when compared to each other in syncytialised BeWo cells although a similar pattern of expression to non-syncytialised BeWo cells and JEG-3 cells was seen.

Our data regarding the gene expression of the three estrogen receptors ERα, ERβ and GPR30 in non syncytialised BeWo cells as well as JEG-3 cells has shown that the dominant receptor expressed in these cells is ERα. The gene expression of this receptor is significantly higher than the gene expression of both other receptors in both cell lines. Furthermore, GPR30 was also expressed in both cell lines and expression was significantly higher in both cell lines when compared to ERβ. These differences in gene expression were also apparent in syncytialised BeWo cells, although not as markedly, as there was no significant difference in expression between ERs.

3.2.2 Quantification of ERα, ERß and GPR30 at protein level in BeWo and JEG-3 cell lines in monolayer

Quantification of Estrogen Receptors ERα, ERß and GPR30 in BeWo and JEG-3 cell lines ERα and ERß expressions were quantified by immunostaining and imaging with the Amnis Imaging Flow Cytometer (ImageStream®). Using Imagestream®, the A549 cell line was used as a positive control, as these cells are known to express all three estrogen receptors (Fan et al., 2017a; Niikawa et al., 2008; Zhu, Huang, Wu, Wei, & Shi, 2016).

3.2.2.1 ImageStream®

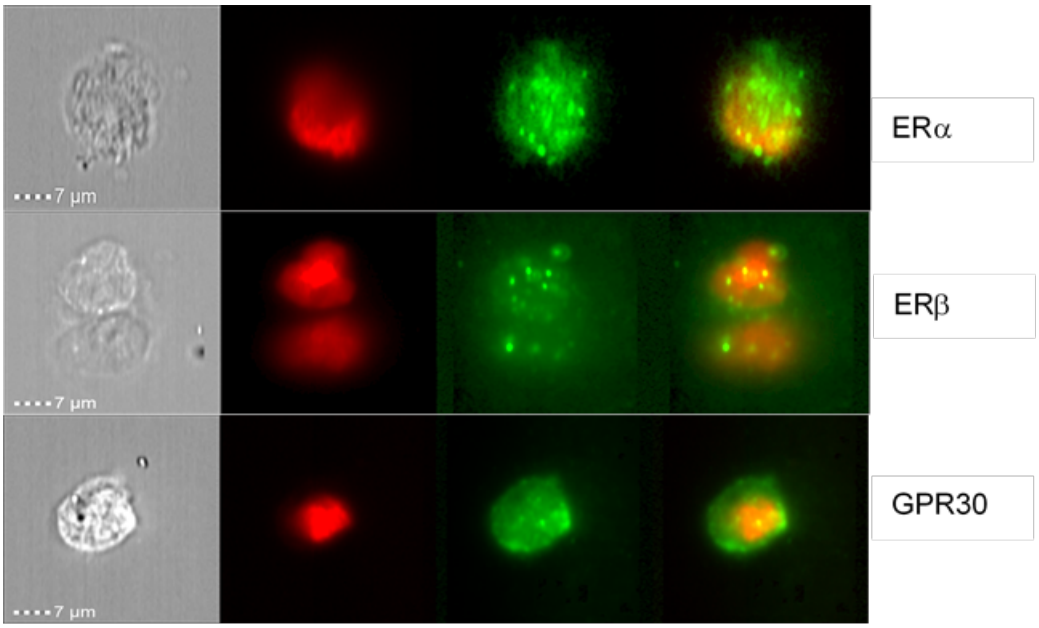

Figure 3.4 Estrogen receptor staining of BeWo cells using ImageStream®. Grey panel: brightfield image. Red: DRAQ5 nuclear stain. Green: Alexa Flour® secondary antibody stain. ERα and ERβ receptors show a more nuclear staining pattern, whereas GPR30 can be found surrounding the nucleus and on the cell membrane. There appears to be less ERβ than ERα.

When using image stream as a method for detecting ERs, we were able to find all three receptors present in BeWo cells. As with gene expression levels, there was more ERα and GPR30 than ERβ on the protein level. In BeWo cells there was a more nuclear staining pattern to the nuclear ERs ERα and ERβ, whereas the membrane receptor GPR30 showed a more peripheral staining pattern.

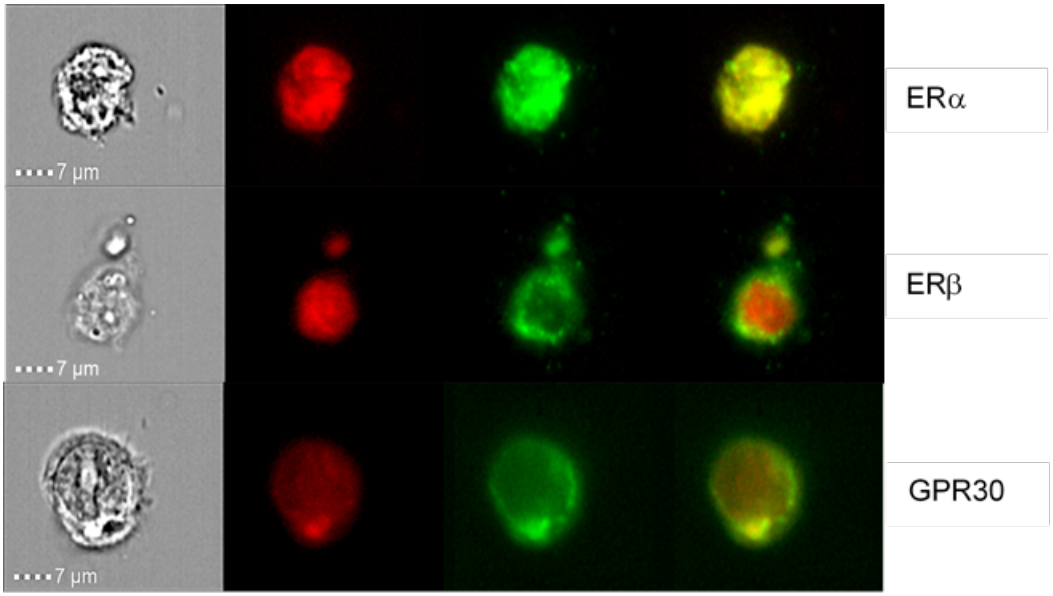

Figure 3.5 Estrogen receptor staining of JEG-3 cells for estrogen receptors. Grey panel: Brightfield. Red: DRAQ5 nuclear stain. Green: Alexa Fluor® secondary antibody stain. ERα shows a more nuclear staining pattern, whereas GPR30 and ERβ can be found surrounding the nucleus and on the cell membrane. There appears to be less ERβ than ERα.

In JEG-3 cells, all three receptors were present. Staining with Alexa Fluor® showed that the most dominant receptor seemed to be ERα, which was in line with previous gene expression studies. This receptor showed a more nuclear staining pattern, whereas ERβ and GPR30 showed more staining on the cell membrane. This ER staining pattern was slightly different than in BeWo cells, where both ERα and ERβ were found within the nucleus, and only GPR30 was found on the cell membrane.

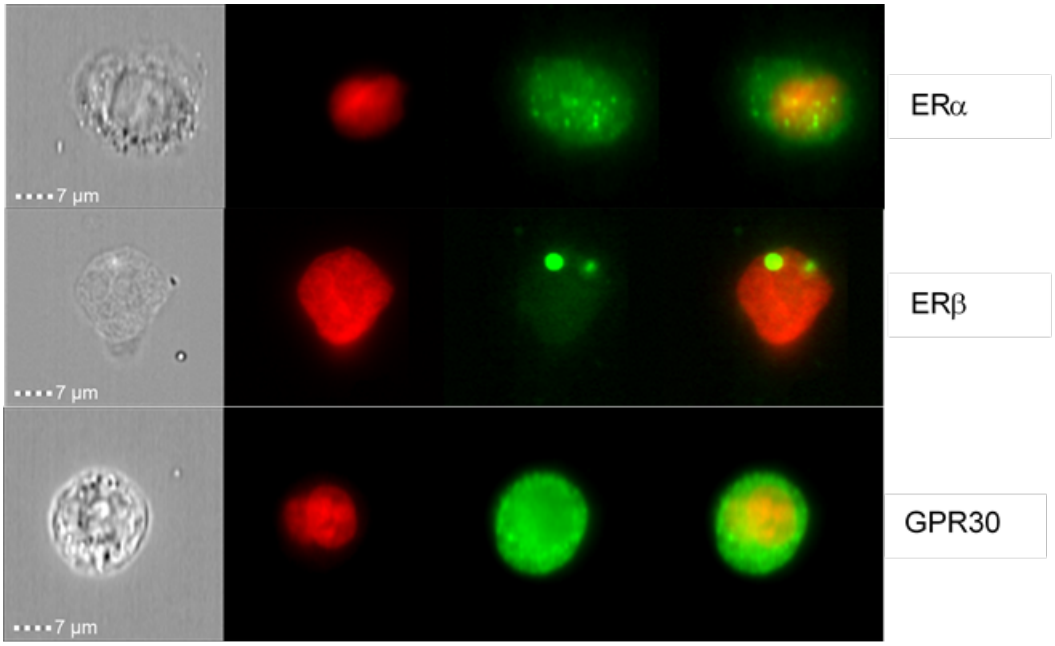

Figure 3.6 Estrogen receptor staining of A549 cells for estrogen receptors. Cells were chosen as a positive control. Grey panel: Brightfield. Red: DRAQ5 nuclear stain. Green: Alexa Fluor® secondary antibody stain. ERα shows a more nuclear staining pattern, whereas GPR30 can be found surrounding the nucleus and on the cell membrane. There is a lack of ERβ staining.

The A549 cell line was chosen as a positive control for ER staining, as presence of all three ERs in the cell line has been documented (Fan et al., 2017; Ivanova, Mazhawidza, Dougherty, & Klinge, 2010; Zhu et al., 2016). In this cell line, ERα appears to be localized more within the nucleus, whereas GPR30 has more of a staining pattern on the cell membrane. There appears to be a lack of ERβ staining.

3.2.2.2 Immunofluorescent detection of estrogen receptors in BeWo and JEG-3 cell lines

In order to further assess the presence and cellular distributions of estrogen receptors in BeWo and JEG-3 cell lines, immunofluorescent staining was employed and cells were imaged.

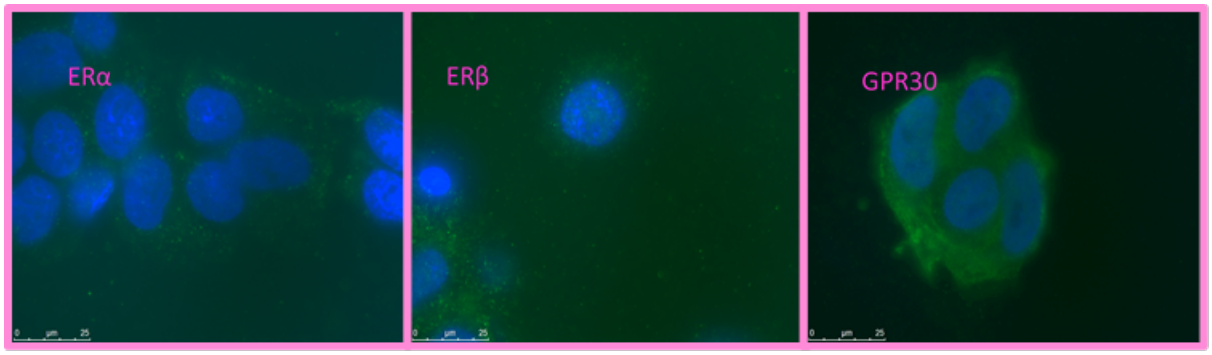

Figure 3.7 Immunostaining of BeWo cell line for estrogen receptors, merged images. Green: receptor, blue: DAPI nuclear stain. Presence of ERα and ERβ is noted and these receptors appear to be evenly distributed throughout the cell as well as being seen in the nucleus. GPR30 is a membrane-bound receptor and therefore staining is seen to be most prominent along the outside of the cell, and not in the nucleus.

Immunofluorescent staining of BeWo cells showed the presence of all three ERs within the cell line. There appeared to be a similar staining intensity of ERα and ERβ as well as GPR30 staining. ERα and ERβ have a more nuclear distribution whereas GPR30 appears to be localized more to the cellular membrane.

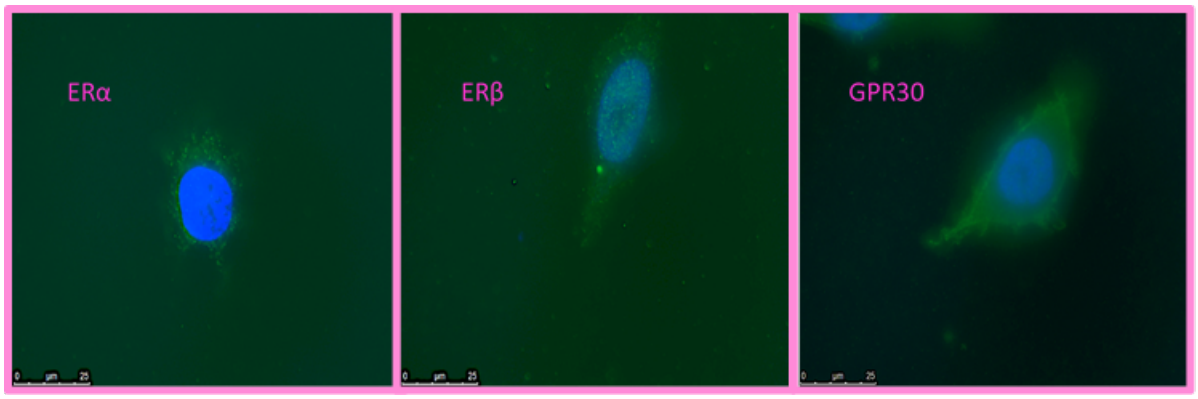

Figure 3.8 Immunostaining of JEG-3 cell line for estrogen receptors, merged images. Green: receptor, blue: DAPI nuclear stain. Presence of ERα and ERβ is noted and these receptors appear to be evenly distributed throughout the cell as well as being seen in the nucleus. GPR30 is a membrane-bound receptor and therefore staining is seen to be most prominent along the outside of the cell, and not in the nucleus.

Immunofluorescent staining of the JEG-3 cell line also showed the presence of all three ERs. As with BeWo cells, there seemd to be a similar staining intensity of all three receptors, and ERα and ERβ showed a nuclear distribution whereas GPR30 was mostly localized along the cellular membrane.

These data show the protein expression of estrogen levels in both the BeWo and JEG-3 cell line. All three receptors are expressed in both cell lines, with a more nuclear distribution in ERα and ERβ, and a more membrane bound pattern of expression for GPR30.

3.2.3 Validation of Syncytialisation of BeWo cell line

BeWo cells were treated with 8-bromo-cAMP for 72 hours in order for them to fuse and form syncytia, making these cells more representative of 2nd and 3rd trimester placental cells. E-cadherin was visualized through immunofluorescence as a marker of cell membrane borders. As trophoblasts fuse to become syncytiotrophoblasts, giant cells containing multiple nuclei and one surrounding cell membrane develop, and E-cadherin around cells is lost (Rebut-Bonneton, Boutemy-Roulier, & Evain-Brion, 1993). With immunofluorescence we have shown that cells lose E-cadherin after treatment with 8-Br-cAMP when compared to controls (Figure 3.11). To compare proliferation rates between syncytialised and unsyncytialised BeWo cells, proliferation rates were measured using the Countess™ automated cell counter system. Cells were grown for 72 hours in the absence or with the addition of 8-Br-cAMP. It is known that after differentiation from cytotrophoblast to syncytiotrophoblast, the syncytiotrophoblast does not divide or proliferate. Therefore, we would expect a lower proliferation rate in syncytialised BeWo cells when compared to non-syncytialised BeWo cells as well as lower viability due to a decrease in growth. Furthermore, syncytiotrophoblasts are more fully endocrine active than cytotrophoblasts. We therefore assessed the expression of β-HCG and estrogen by these cells, and compared them to their unsyncytialised state. Furthermore, we assessed gene expression of syncytin-2 in both non-syncytialised and syncytialised BeWo cells, expression of which correlates with differentiation into syncytia (Vargas et al., 2009). Lastly, the gene expression and protein levels of ERs was assessed in syncytialised cells, as studies have shown an increase in ERα and ERβ in syncytiotrophoblast and closer to term (Kim, Park, Lee, Joo, & An, 2016).

3.2.3.1 Immunofluorescent staining of E-Cadherin in syncytialised BeWo cells

E-Cadherin staining in BeWo cells after treatment with 8-Br-cAMP shows that BeWo cells have syncytialised, as when compared to controls, cell membranes have broken down in order for cells to fuse and form large, multinucleated syncytia, the more endocrine active component of the placenta (Cole, 2012; Costa, 2016).

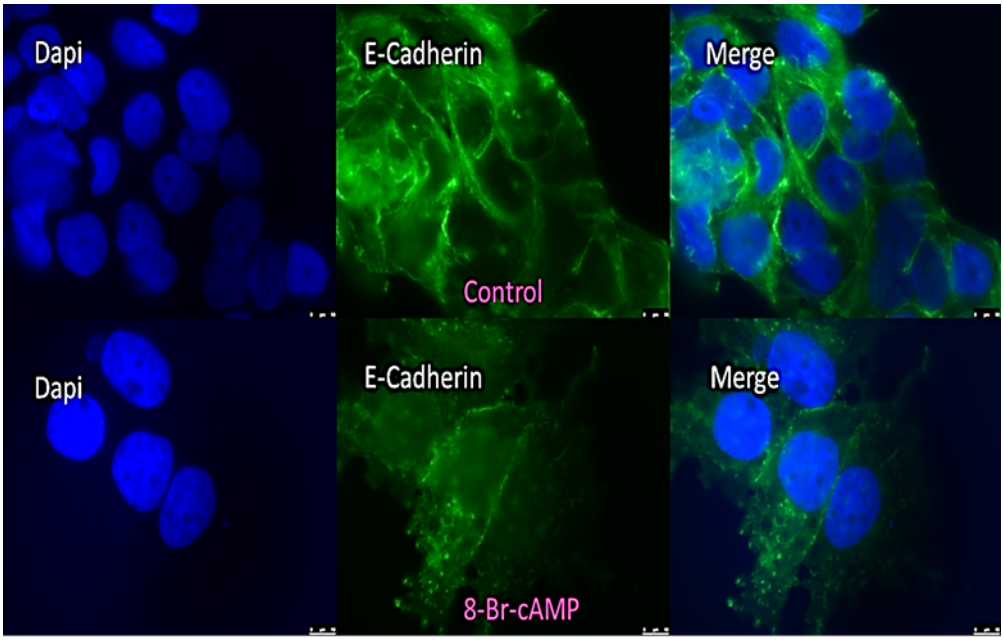

Figure 3.9 Immunofluorescent staining of E-Cadherin in BeWo cells treated with 8-Br-cAMP in order to syncytialise. Green: E-Cadherin. Blue: DAPI nuclear stain. Cells depicted in the bottom row have been treated with 8-Br-cAMP for 72 hours, cells depicted in the top row have not. As cells treated with 8-Br-cAMP fuse to become syncytia (amorphous and multinucleated cells), cell walls break down and lose E-Cadherin.

3.2.3.2 Proliferation

In order to further investigate whether cells treated with 8-Br-cAMP had syncytialised, it was necessary to compare proliferation between non-syncytialised and syncytialised BeWo cells, as syncytialised cells are differentiated and proliferation decreases. (Huppertz, Frank, Kingdom, Reister, & Kaufmann, 1998). Non-syncytialised and syncytialised BeWo cells were grown for 24 hours and cell numbers as well as dead cells were compared using the Countess® system. Our data showed a higher number of non-syncytialised BeWo cells after 24 hours, with 86% of cells being live compared

to 70% of cells being lived in non-syncytialised cells, indicating more growth in non-syncytialised cells.

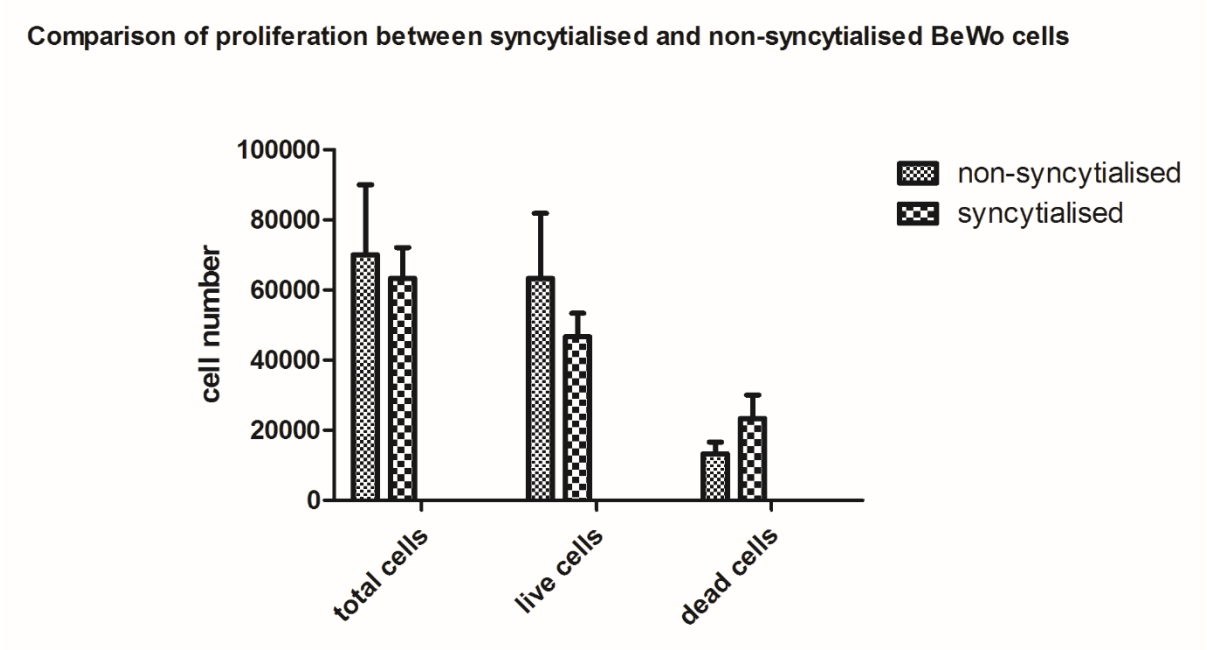

Figure 3.10 Comparison of proliferation between non-syncytialised and syncytialised BeWo cells after 24 hours. Syncytialised cells were treated with 8-Br-cAMP for 72 hours prior to proliferation being assessed. After 24 hours, syncytialised BeWo cells showed slightly less total cells than non-syncytialised BeWo cells, with 70% of cells being live compared to 86% of cells being live in non-syncytialised BeWo cells.

3.2.3.3 Hormone Secretion

As previously mentioned, syncytiotrophoblast is the more endocrine active cell type when compared to cytotrophoblast. Therefore, we measured hormone (β-hCG and E2) levels in conditioned media of non-syncytialised and syncytialised BeWo cells grown for 24 hours. Our findings of significantly increased hormone secretion (β-hCG $p=0.0108$; E2 $p=0.0042$) in cells treated with 8-Br-cAMP confirms successful syncytialisation of BeWo cells.

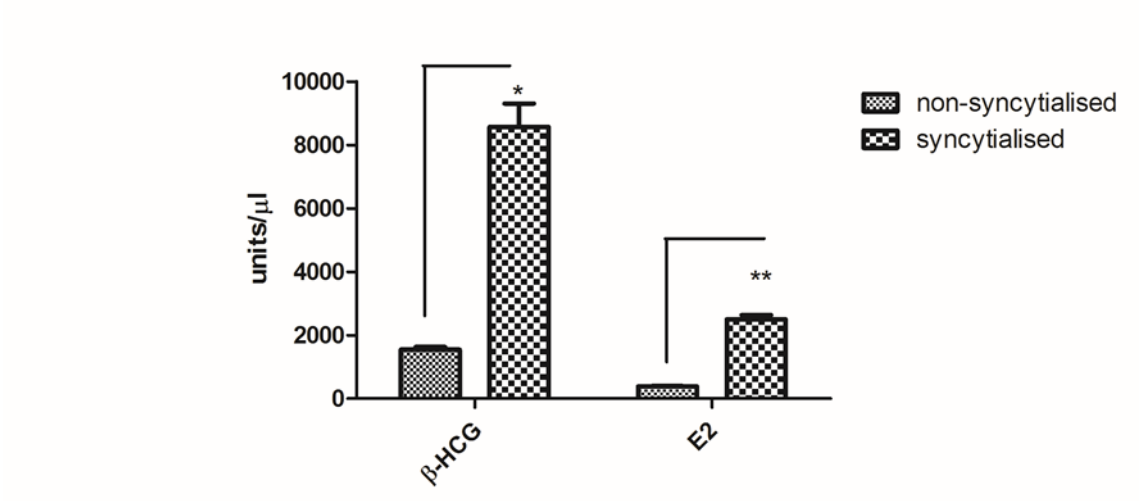

Figure 3.11 Secretion of estrogen (E2) and β-HCG in conditioned media of non-syncytialised and syncytialised BeWo cells grown for 24 hours. Expression of β-hCG was significantly upregulated (p=0.0108) in syncytialised BeWo cells and expression of E2 was also significantly upregulated (p=0.0042) in syncytialised BeWo cells compared to non-syncytialised BeWo cells.

3.2.3.4 Expression of Syncytin

Syncytin-2 is an envelope protein derived from human endogenous retroviruses (HERVs) and plays an important role in trophoblast syncytialisation (Vargas et al., 2009) and can therefore be seen as a marker of syncytialisation in BeWo cells. In order to asses whether syncytialisation had taken place in BeWo cells treated with 8-br-cAMP, we measured gene expression of syncytin-2 in BeWo cells treated with 8-br-cAMP for 72 hours compared to non-treated cells. Syncytin-2 showed a more than 2-fold upregulation in syncytialised cells. Taking into account that one study found that on average, the syncytialisation rate of BeWo cells treated with forskolin is 10% (Kudo et al., 2003), the level of upregulation is an indicator of syncytialisation having taken place.

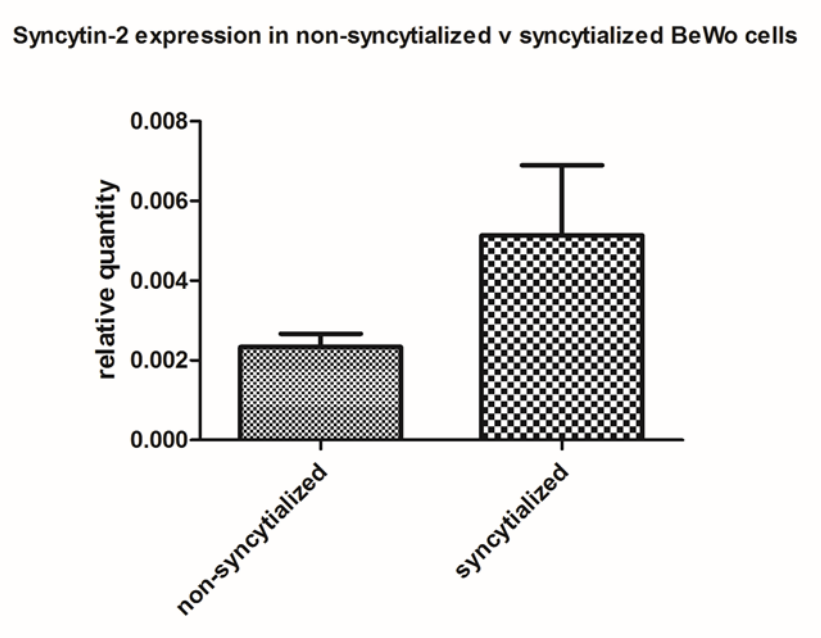

Figure 3.12 Expression of syncytin-2, a marker of syncytialisation, in non-syncytialised and syncytialised BeWo cells. There is a more than 2-fold increase in syncytin-2 in cells treated with 8-Br-cAMP. Relative quantities are levels of gene of interest in relation to quantities of housekeeping gene (TOP1).

3.2.3.5 Estrogen receptor expression in syncytialised cells

As before, ER gene expression levels were measured in syncytialised BeWo cells and compared to levels in non-syncytialised cells to assess whether ER gene expression changed. After syncytialisation, there was an upregulation in gene expression of all ERs, however this was not significant.

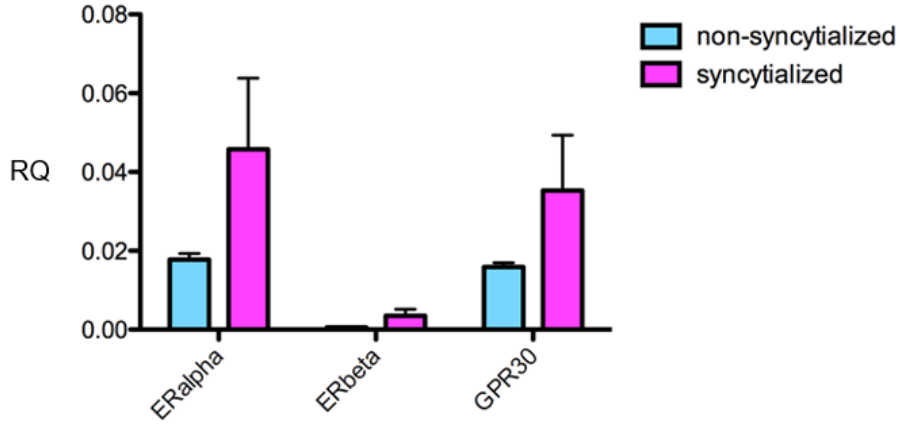

Figure 3.13 All receptors are upregulated in syncytialised BeWo cells compared to non-syncytialised BeWo cells. There was an increase in RQ of ERα from 0.02 to 0.05, an increase in ERβ from 0 to 0.005 and an increase in GPR30 from 0.019 to 0.04. RQ was measured as relative quantity of gene expression compared to housekeeping gene (TOP1).

In order to asses the amount of ER protein present in syncytialised BeWo cells, Immunofluorescent staining was used. As we had previously seen an upregulation of ER gene expression in syncytialised cells compared to non-syncytialised BeWo cells (Figure 3.13), we were expecting upregulation of all three receptors on the protein levels as well. Figure 3.14 shows immunostaining of ERα, ERβ and GPR30, where there appears to be an increase in staining intensity, when compared to non-syncytialised BeWo cells (Figure 3.7).

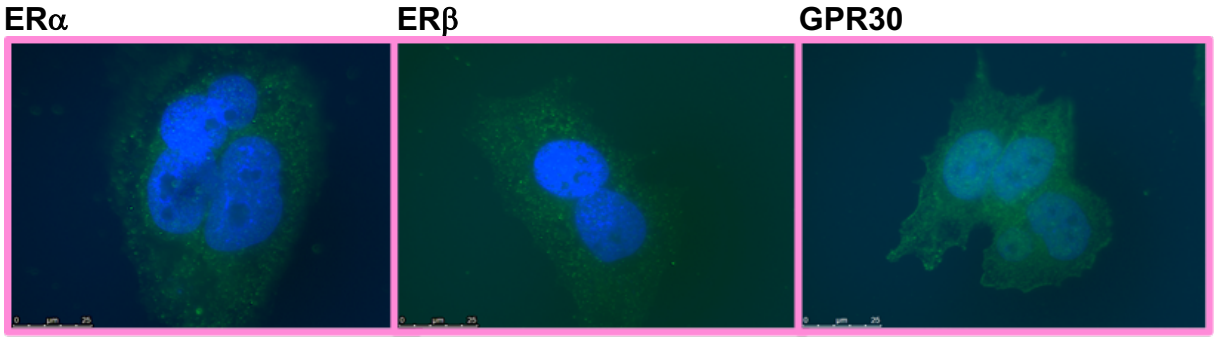

Figure 3.14 Immunostaining of syncytialised BeWo cell line for estrogen receptors, merged images. Green: receptor, blue: DAPI nuclear stain. When compared to Immunostaining of non-syncytialised cells (Figure 3.7), there appears to be an increase in receptor density. As before, ERα and ERβ show a more nuclear staining pattern, whereas GPR30 stining is more focused around the cell membrane.

In terms of syncytialisation, we have shown that E-cadherin, a cell surface marker, is decreased markedly in BeWo cells treated with 8-Br-cAMP for 72 hours, providing evidence for cell fusion, which is typical for syncytium. Furthermore, cells treated with 8-Br-cAMP showed less proliferation than untreated cells, which is expected in cells that are terminally differentiated. We have also shown that there is a significant increase in both E2 and β-hCG in treated cells indicating a switch from cytotrophoblast to syncytiotrophoblast, the more endocrine active cell. Syncytin-2, a marker of syncytialisation, was also more highly expressed in treated BeWo cells. Lastly, qPCR and immunostaining showed an upregulation of all ERs on the gene transcription as well as the protein level.

3.3 Discussion

With the demonstration that our receptors are present in the cell lines tested, and with the finding that ERα is the most abundant receptor, our results are partly in line with studies that report ERα as the primary estrogen receptor in the placenta (Bukovsky et al., 2003; Schiessl et al., 2006; Yin et al., 2013) with an increase in ERβ expression in the later stages of placental development. By showing that there is an upregulation of ERs in syncytialised cells, which reflect placenta closer to term, our data is in agreement with studies that have found that translational levels of ERα and ERβ were higher in later stages of gestation in humans (Fujimoto et al., 2005; Kim et al., 2016), and since syncytialised BeWo cells are reflective of more mature placenta, our findings point to successful syncytialisation after treatment with 8-Br-cAMP. Furthermore, we have demonstrated the differentiation of BeWo cytrotrophoblast cells into BeWo syncytiotrophoblasts using 8-Br-cAMP.

As cytotrophoblasts fuse to become syncytiotrophoblasts, their individual cell membranes degrade and expression of cell membrane bound proteins that mediate cell-to-cell interaction such as E-cadherin, decreases (Candelier et al., 2013; Rebut-Bonneton et al., 1993). Using E-cadherin as a marker for cell fusion and loss of cell membrane, we have demonstrated a decrease in staining intensity of E-cadherin when comparing non-syncytialised to syncytialised BeWo cell lines using immunofluorescence. Furthermore, we have shown that BeWo cells treated with 8-Br-

cAMP lose the capacity to proliferate, and therefore become less viable than untreated cells, another indicator that cells have indeed fused and become syncytia. We have also shown that syncytialised cells significantly expressed more estrogen and β-hCG than non-syncytialised cells. Studies have shown that syncytiotrophoblast is the main endocrine cell when compared to cytotrophoblast (Lunghi et al., 2007), and that it is the main cellular component in term placenta (Jiang et al., 2018). From pregnancy towards term, the hormonal function of the placenta gradually increases towards a production of chorionic somatomammotropic hormone (hCS) of 1-3g/day at the end of pregnancy, due to the gradual increase of syncytiotrophoblast mass (Evain-Brion & Malassine, 2003).

To further validate the differentiation of BeWo cells from cytotrophoblasts to syncytiotrophoblasts, gene expression of syncytin-2, a marker of differentiation that is placenta-specific and crucial for cell fusion to take place (Vargas et al., 2009), was measured in both 8-Br-cAMP treated and non-treated BeWo cells. Our studies showed a 2-fold upregulation of syncytin-2 in 8-Br-cAMP treated cells. Although this fold change might not be significant, it must be taken into account that syncytialisation after 8-Br-cAMP treatment only syncytialises a fraction of cells. In cells treated with forskolin for 48 hours, 10% of cells were shown to be fused (Kudo et al., 2003b), and similar rates could be assumed with 8-Br-cAMP treatment.

Overall, our data suggest that both BeWo and JEG-3 cell lines can be used as a valid model to study the effects of BPA at placental level, as receptors by which this compound has the potential to act are present in these cell lines to an extent which is reflective of human placenta *in vivo.* Furthermore, transforming BeWo non-syncytialised cells into syncytialised cells has given the opportunity to test compounds in cells representative of earlier stages of placental development and the differentiated (syncytialised) cells that make up most of the organ closer to the end of pregnancy. Not only do cells show that they have been syncytialised morphologically, but behave in a similar way to in *vitro* placental tissue regarding hormone secretion, receptor expression, and proliferation. Taking these factors into account, experiments performed on the cell line will accurately reflect placental physiology when it comes to effects that are mediated by estrogen receptors. We have therefore chosen BeWo

cells for future experiments in this study, in order to test the effects of BPA on both cells that have been syncytialised and non-syncytialised cells.

Chapter 4

Role of BPA in human placental cells *in vitro*

4.1 Introduction

As mentioned, BPA is an endocrine disruptor known to exert its function via estrogenic activity using estrogen receptors. Depending on the tissue, the mechanism of binding and the receptor, BPA has the potential to exert a multitude of varying effects, ranging from estrogen mimicking activity to anti-estrogenic activity. BPA has been shown to have an estrogenic effect in the breast cancer cell line MCF-7 (Krishnan et al., 1993) as well as rat vagina and uterus (Kurosawa et al., 2002; Steinmetz et al., 1998). Although BPA binds to classical nuclear estrogen receptors with much lower affinity than E2, estrogen-mimicking effects have been demonstrated even with low levels of BPA (Alonso-Magdalena et al., 2012; Fang et al., 2000). Other studies have shown that higher levels can cause an anti-estrogenic effect in tissues. Because of the varying mechanism of action of BPA, depending on metabolism, exposure, tissue environment and receptor expression, there is no cohesive answer to the question of the effect of BPA in human cells. In this chapter, we aim to elucidate the effects and mechanisms of action of BPA in human placental cells, by studying intra-cellular (phospho-) targets affected by BPA-treatment of cell lines.

Nuclear ERs mediate DNA transcription, whereas the receptors themselves are ligand-dependent transcription factors, which recruit either coactivating or corepressing factors, thereby either silencing or inducing genes (Huang et al, 2010, Mattison et al, 2014). Nuclear ERs are found in varying tissues in the human body, mainly in female reproductive tissues such as the mammary gland, ovary and uterus, bone, testis, prostate, bladder, colon, immune system and adipose tissue (Farzaneh & Zarghi, 2016), with different distributions of ERα and ERβ.

Besides the classic nuclear receptors ERα and ERβ, a more recently discovered G-protein-coupled ER, known as GPR30 or GPER, has been shown to bind BPA. GPCRs mediate fast intracellular responses, such as activation of second messengers, phosphorylation of kinases and ion mobilization (Prossnitz & Arterburn,

2015). Estrogenic activation of GPR30 results in a multitude of intracellular effects, such as activation of ERK1/2 and the PI3K/AKT pathway, vascular dilation via production of eNOS, the regulation of potassium channels and the mobilization of Ca^{2+} (Filardo et al, 2000; Revankar et al, 2005; Meyer et al, 2012; Lindsey et al, 2014). These cellular changes have the potential to affect tissue physiology, and play a role in the functionality of the organ.

The mechanism of action of BPA in placental tissue specifically is not well documented, and studies about its primary binding partner and effect have been conflicted. Studies have shown that BPA binds weakly to ERα and ERβ with similar affinities for both receptors (Prossnitz & Arterburn, 2015; Blair et al, 2000; Kuiper et al, 1998), but also that BPA signals primarily through mediation by ERα as opposed to ERβ (Hewitt & Korach, 2011). Numerous studies have demonstrated that effects of BPA are mediated at least in part by GPR30 or GPER (Prossnitz & Arterburn, 2015) and that the binding affinity to GPR30 is 8-50 times higher than for the nuclear receptors ERα and ERβ (Thomas & Dong, 2006; Blair et al, 2000). In many cases, BPA was shown to stimulate proliferation of cells in low doses via the GPR30 receptor in different types of tissues, via phosphorylation of ERK1/2 (Chevalier et al, 2012; Sheng et al, 2014; Li et al, 2017; Sauer et al, 2017), however studies with higher levels of BPA exposure ranging in the micromolar region have shown opposite effects (Kidani et al, 2017). None of these studies, however, have examined the non-genomic effects of BPA on trophoblastic cell lines and how these effects are mediated.

As mentioned before, there is little data delineating the non-genomic effects of BPA on trophoblastic cell lines used as models of the human placenta, as well as mapping in detail the expression of all ERs at gene and protein level. It is however crucial to understand how BPA alone and in combination with other factors can work to change placental physiology, which in turn has the potential to affect pregnancy, fetal survival and placental viability.

The main aim of this chapter is to elucidate how treatment of the placental cell line BeWo with low levels of BPA affects changes in intracellular target phosphorylation on the protein level, the mechanism of action of BPA in terms of receptor binding, and

whether ER expression is affected by BPA treatment. Therefore, this chapter has been structured into three aims:

1. Elucidate the effect of 3 nM and 10 nM BPA on the phosphorylation status of ERK1/2, p38 and AKT *in* vitro at the protein level. These specific phospho-targets have been chosen as effectors of Estrogen signaling.

2. Determine the mechanism of action by which BPA influences the phosphorylation of certain intracellular targets, by employing the use of estrogen receptor antagonists as well as MAPK and AKT inhibitors.

3. Study the gene expression level of estrogen receptors ERα, ERβ and GPR30 before and after treatment with 3 nM and 10 nM BPA to determine whether BPA has an effect on the expression of estrogen receptors.

4.2. Results

4.2.1 Effect of BPA on the phosphorylation status of p38, AKT and ERK1/2 *in* vitro

In order to assess the short-term effect of BPA on BeWo cell lines, cells were treated with BPA in physiologically relevant concentrations of 3 nM and 10 nM for 5, 15, 30 and 60 minutes. After this, expression of specific phospho-targets was measured. These were chosen due to their relevance in estrogen signaling.

When BeWo cells were treated with 10 nM BPA for 5 minutes, levels of phosphorylated p38 (p-p38) were significantly increased compared to non-treated samples ($p<0.05$) (Figure 4.1). This significance was not seen in cells treated with 3 nM treated samples. In Western blot images, it can be seen that levels of housekeeping gene (GAPDH) as well as levels of total p38 (phosphorylated and non-phosphorylated) have remained constant, while band intensity of p-p38 in 10 nM BPA treated samples has increased. Interestingly, when BeWo cells were treated with BPA for 15 minutes, we saw a non-significant decrease in p-p38 which was most marked in 10 nM treated cells. There

was a significant increase ($p<0.05$) in p-p38 protein expression after treatment with 10 nM BPA for 30 minutes (Figure 4.3) but not when treated with 3 nM BPA. This increase was seen in bands whereby GAPDH levels remained constant as well as total p-38 levels. After 60 minutes of 3 nM and 10 nM BPA treatment (Figure 4.4), p-p38 levels were significantly increased in both 3 nM treated cells ($p<0.05$) as well as 10 nM treated cells ($p<0.01$). Bands showed a constant level of GAPDH expression as well as total p38 expression with a marked increase in band thickness in 3 nM and 10 nM treated cells.

p-AKT expression was also measured in BeWo cells treated with 3 nM and 10 nM BPA over the same time points as previously discussed. Figure 4.5, 4.6 and 4.7 show that treatment 3 nM and 10 nM BPA for 5, 15 and 30 minutes, respectively, did not result in a significant increase in p-AKT expression. After 60 minutes of BPA treatment (Figure 4.8), there was a significant increase in p-AKT expression ($p<0.05$) in 10 nM treated BeWo cells, which is also shown in the increased thickness of the 10 nM treated bands, when compared to controls. GAPDH and total AKT levels remained the same with treatment.

p-ERK1/2 expression was also measured at the same treatment levels and time points, however figures 4.9, 4.10, 4.11 and 4.12 show that there was no significant upreglation of expression of p-ERK1/2 in BeWo cells treated with either 3 nM or 10 nM BPA.

4.2.1.1 p-p38 expression

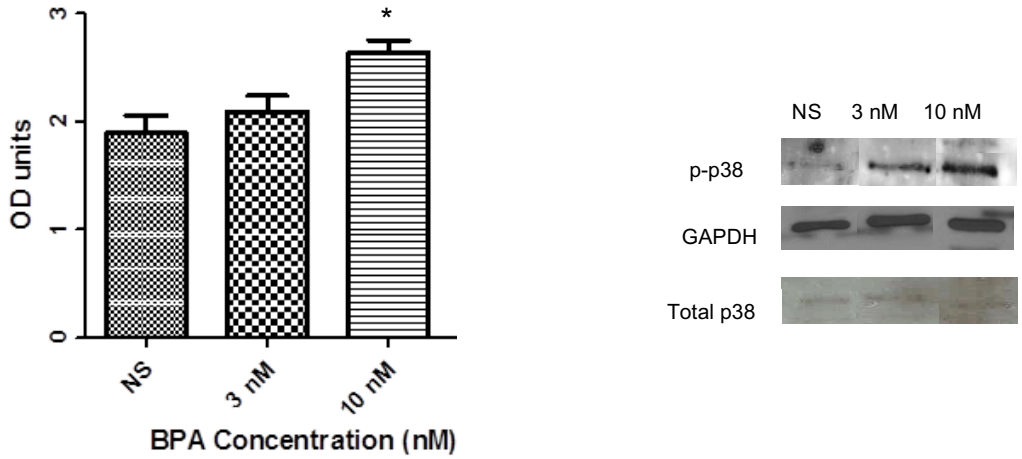

Figure 4.1. relative amount of p-p38 (phosphorylated p38) after 5 minutes of BPA treatment. NS = no supplement. OD units = optical density units. Graph and images show that the treatment of BeWo cells with 10 nM BPA significantly increased the protein expression of p-p38 after 5 minutes ($p<0.05$). Both protein expression of housekeeping gene GAPDH as well as the expression of total p38 have remained constant.

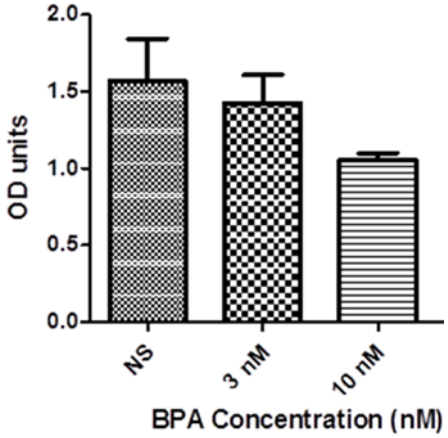

Figure 4.2. relative amount of p-p38 after 15 minutes of BPA treatment. There was no significant increase in the expression of p-p38 after 15 minutes of BPA treatment.

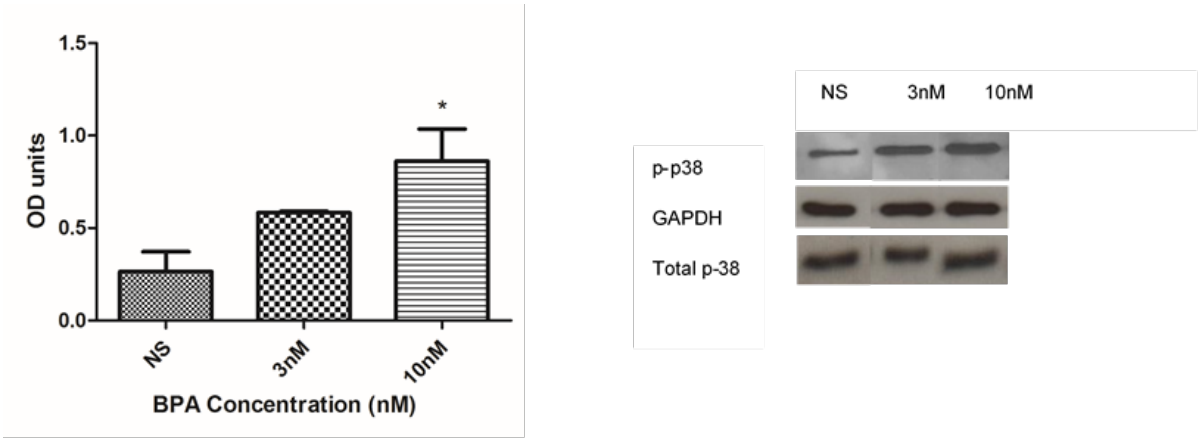

Figure 4.3. relative amount of p-p38 after 30 minutes of BPA treatment. Graph and images show that the treatment of BeWo cells with 10 nM BPA significantly increased the protein expression of p-p38 after 30 minutes ($P<0.05$). Both protein expression of housekeeping gene GAPDH as well as the expression of total p38 have remained constant.

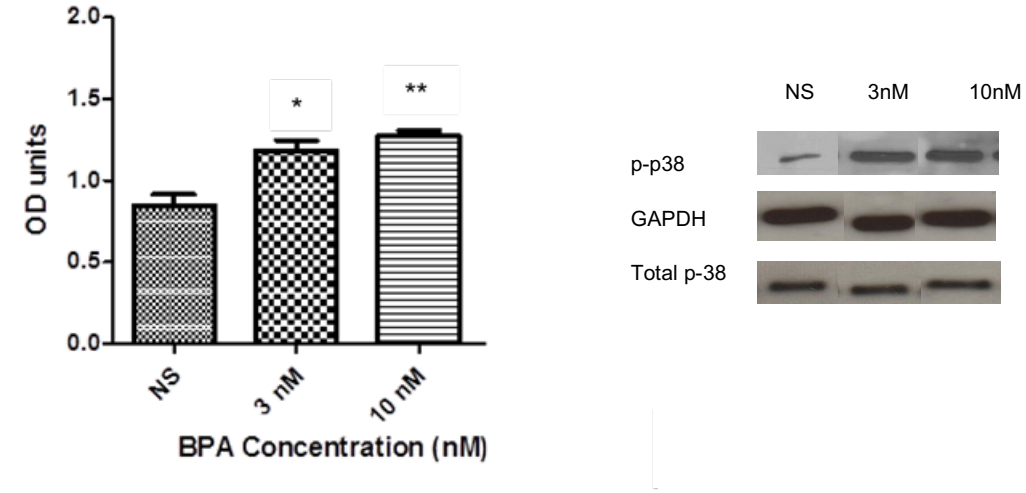

Figure 4.4. relative amount of p-p38 after 60 minutes of BPA treatment. Graph and images show that the treatment of BeWo cells with 3 nM and 10 nM significantly increased the protein expression of p-p38 after 60 minutes ($p<0.05$ for 3 nM and $p<0.01$ for 10 nM). Both protein expression of housekeeping gene GAPDH as well as the expression of total p38 have remained constant.

4.2.1.2 p-AKT expression

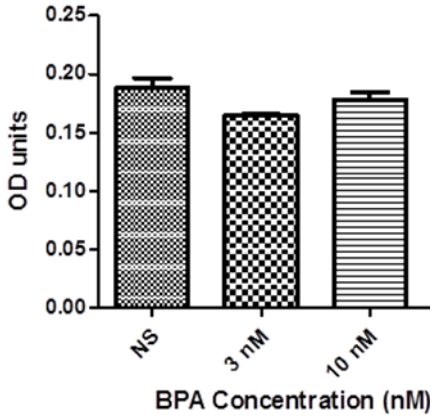

Figure 4.5. relative amount of p-AKT after 5 minutes of BPA treatment. There was no significant increase in the expression of p-AKT after 5 minutes of BPA treatment.

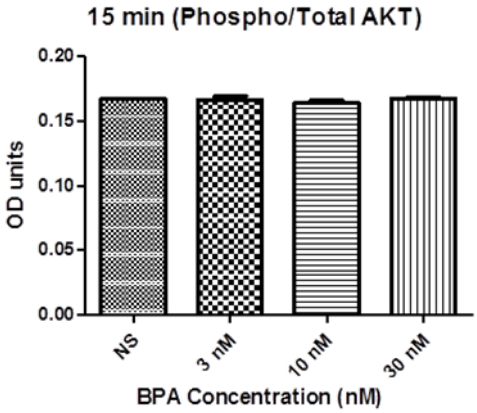

Figure 4.6. relative amount of p-AKT after 15 minutes of BPA treatment. There was no significant increase in the expression of p-AKT after 5 minutes of BPA treatment.

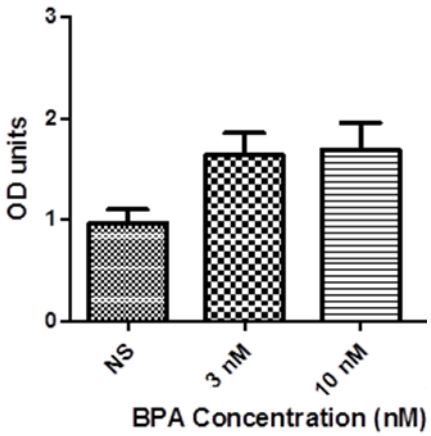

Figure 4.7. relative amount of p-AKT after 30 minutes of BPA treatment. There was no significant increase in the expression of p-AKT after 30 minutes of BPA treatment.

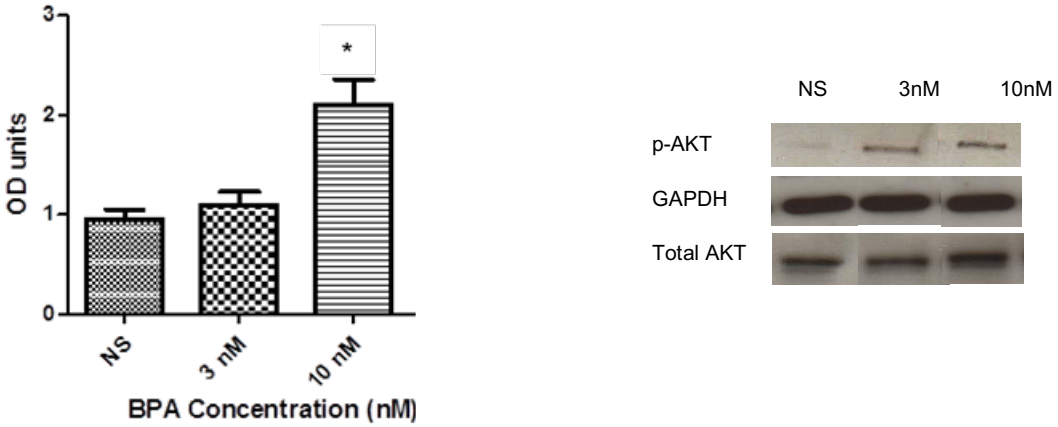

Figure 4.8. relative amount of p-AKT after 60 minutes of BPA treatment. Graph and images show that the treatment of BeWo cells with 10 nM significantly increased the protein expression of p-AKT after 60 minutes ($p<0.05$). Both protein expression of housekeeping gene GAPDH as well as the expression of total AKT have remained constant.

4.2.1.3 p-ERK1/2 expression

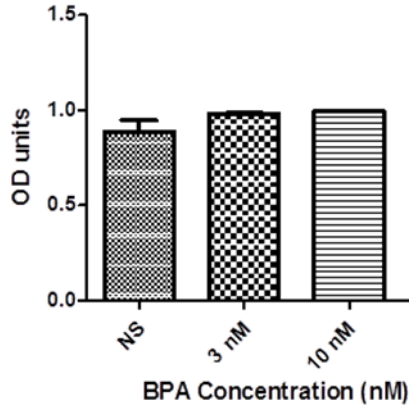

Figure 4.9. relative amount of p-ERK1/2 after 5 minutes of BPA treatment. There was no significant increase in the expression of p-ERK1/2 after 5 minutes of BPA treatment.

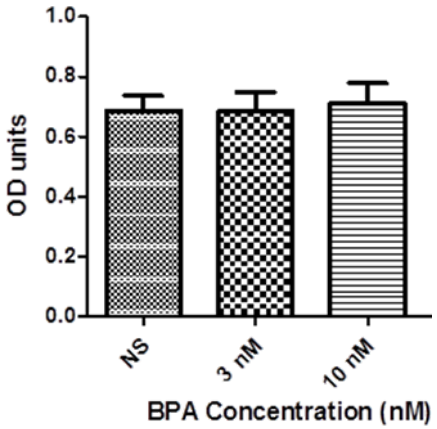

Figure 4.10. relative amount of p-ERK1/2 after 15 minutes of BPA treatment. There was no significant increase in the expression of p-ERK1/2 after 15 minutes of BPA treatment.

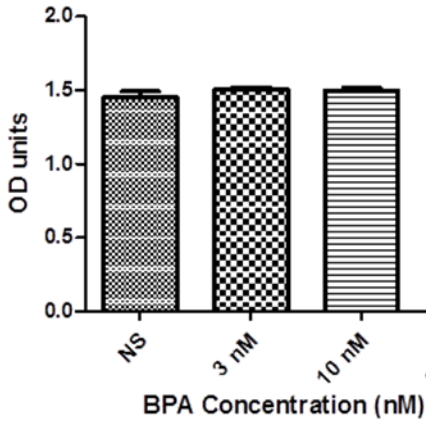

Figure 4.11. relative amount of p-ERK1/2 after 30 minutes of BPA treatment. There was no significant increase in the expression of p-ERK1/2 after 30 minutes of BPA treatment.

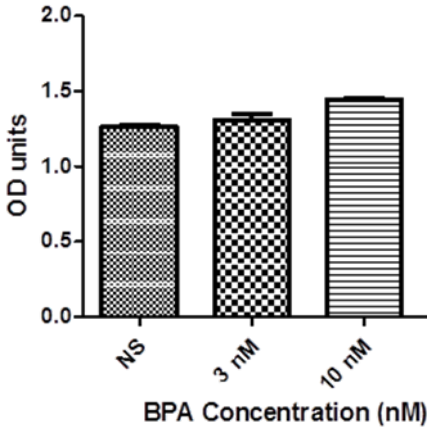

Figure 4.12. relative amount of p-ERK1/2 after 60 minutes of BPA treatment. There was no significant increase in the expression of p-ERK1/2 after 60 minutes of BPA treatment.

4.2.1.4 Immunofluorescent staining

Immunofluorescent staining was performed in order to validate whether protein levels of phosphotargets were upregulated after short-term BPA treatments as previously identified with Western blots. As with Western blots, immunofluorescent staining showed an upregulation of p-p38 (Figure 4.13) when compared to controls after treatment with 10 nM BPA for 60 minutes. Immunostaining of BeWo cells also showed an upregulation of p-AKT (Figure 4.14) after 60 minutes of 10 nM BPA treatment.

Again, similar to Western blot treatment, there was no upregulation of p-ERK1/2 seen after 60 minutes of 10 nM BPA treatment (Figure 4.15).

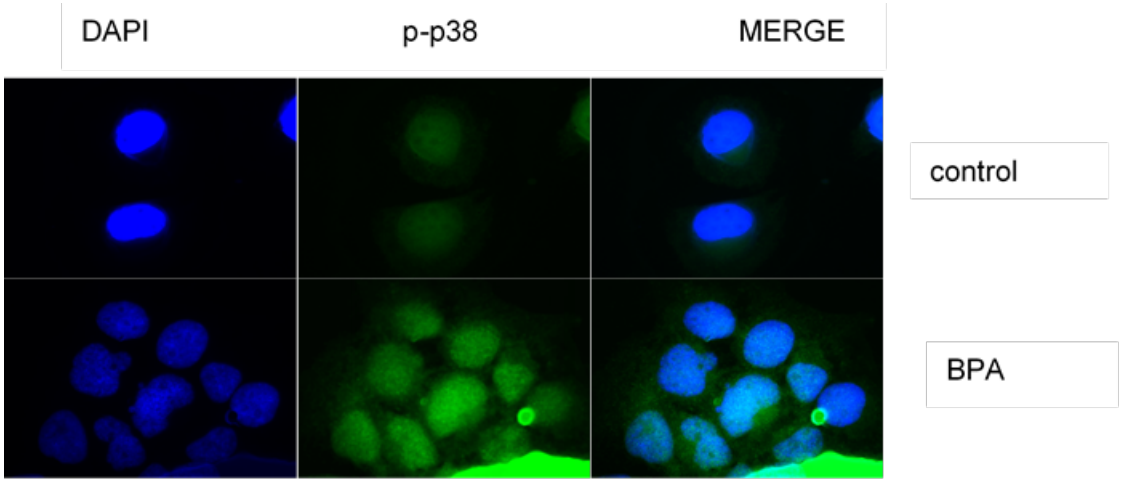

Figure 4.13. Immunostaining of BeWo cells treated with 10 nM BPA for 60 minutes and controls. Immunofluorescent signal shows an upregulation of p-p38 in treated cells compared to controls. P-p38 is located within the cytosol of the cell (green). DAPI = 4′,6-diamidino-2-phenylindole nuclear stain.

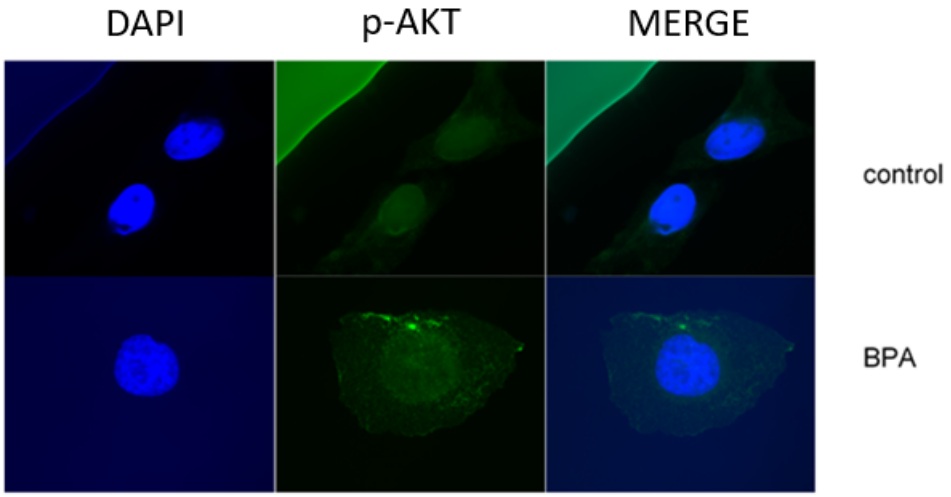

Figure 4.14. Immunostaining of BeWo cells treated with 10 nM BPA for 60 minutes. Immunofluorescent signal shows an upregulation of p-AKT in treated cells compared to controls. P-AKT is located within the cytosol of the cell (green).

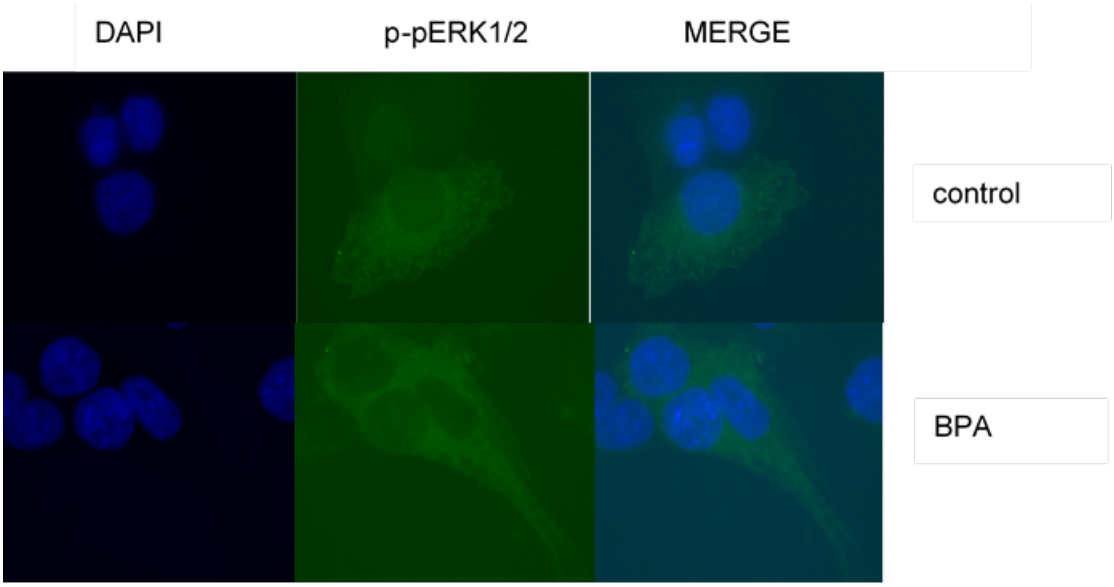

Figure 4.15. Immunostaining of BeWo cells treated with 10 nM BPA for 60 minutes. Immunofluorescent signal shows no upregulation of p-ERK1/2 in treated cells compared to controls. P-ERK1/2 is located within the cytosol of the cell (green).

4.2.2 Dissecting the signalling pathways induced by BPA using receptor antagonists and intracellular signalling inhibitors: effects on cell proliferation

BeWo cells were treated with BPA in order to assess effects of BPA on proliferation of these cells. Furthermore, functional studies were used to investigate the mechanisms of action by which BPA exerts it functions. Cells were treated with 3 nM BPA in the presence or absence inhibitors of AKT (LY294002) and ERK1/2 (UO126) and inhibitors of estrogen receptors (ICI, G-15). LY294002 is a P13K inhibitor which thereby inhibits downstream effectors including AKT (Hu, Zaloudek, Mills, Gray, & Jaffe, 2000; Vlahos, Matter, Hui, & Brown, 1994). U0126 inhibits MAP kinases such as ERK via the direct inhibition of the mitogen-activated protein kinase kinases MEK-1 and MEK-2 (Favata et al., 1998). ICI is a universal estrogen receptor α and β antagonist (Wakeling & Bowler, 1992) and G-15 is an inhibitor of GPR30 (Dennis et al., 2009). Cells were either treated with BPA alone, or in combination with the relevant inhibitor or antagonist, in order to assess how BPA was affecting cell proliferation in BeWo cells.

4.2.2.1 Proliferation of BeWo cells after BPA treatment

When BeWo cells were treated with 3 nM and 10 nM BPA, as well as 30 nM E2 as a positive control, we found that there was a significant ($p<0.05$) increase in proliferation of BeWo cells over 24 hours of 3 nM BPA treatment (Figure 4.16). 10 nM treated BeWo cells and 30 nM E2 treated cells also demonstrated an increase in proliferation but did not reach significance.

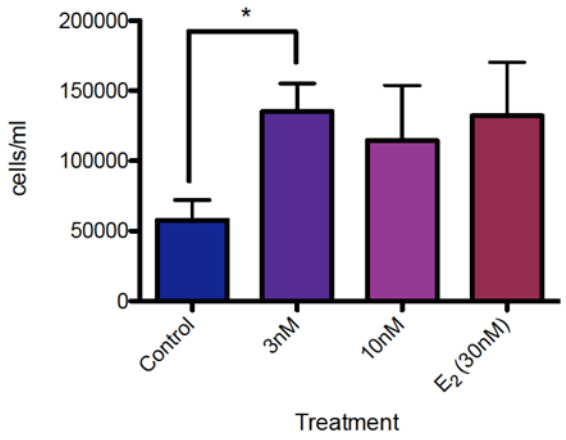

Figure 4.16 Proliferation of BeWo cells treated with 3 nM BPA, 10 nM BPA and 30 nM E2. 3 nM BPA treatment significantly increased cell proliferation compared to controls ($p<0.05$). There was a notable -but no significant- increase in proliferation when cells were treated with 10 nM BPA or 30 nM E2.

4.2.2.2 Proliferation studies using phospho-target inhibitors and estrogen receptor antagonists

As proliferation of BeWo cells was significantly increased after treatment with 3 nM BPA, it was necessary to elucidate by which mechanism this increase of proliferation is mediated. In order to determine mechanisms of action of BPA, the AKT-inhibitor LY294002 as well as the MAPK-inhibitor UO126 and estrogen receptor antagonists ICI and G15 were used with and without BPA treatment. We found that there was a significant decrease in proliferation over 24 hours when treated with 3 nM BPA plus the AKT inhibitor ($p<0.001$), and the AKT inhibitor alone ($p<0.001$) (Figure 4.17), however there was no significant change in proliferation when cells were treated with the MAPK inhibitor (Figure 4.18). When treating BeWo cells with ER antagonists, (Figure 4.19), there was a significant decrease in proliferation over 24 hours when cells were treated with G15+BPA ($p<0.05$). BPA treatment in presence of ICI led to a decrease in cell proliferation but did not reach significance.

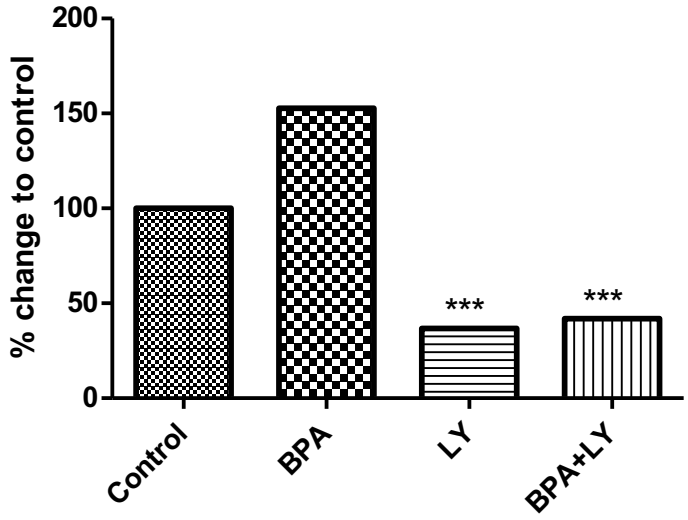

Figure 4.17 BeWo cells were treated with 3 nM BPA in the presence of AKT-inhibitor LY294002, 3 nM BPA, or a combination of both for 24 hours. There was a significant decrease in cell proliferation when cells were treated with LY294002 as well as treatment with BPA + LY294002 when compared to controls and treatment with only BPA ($p<0.01$).

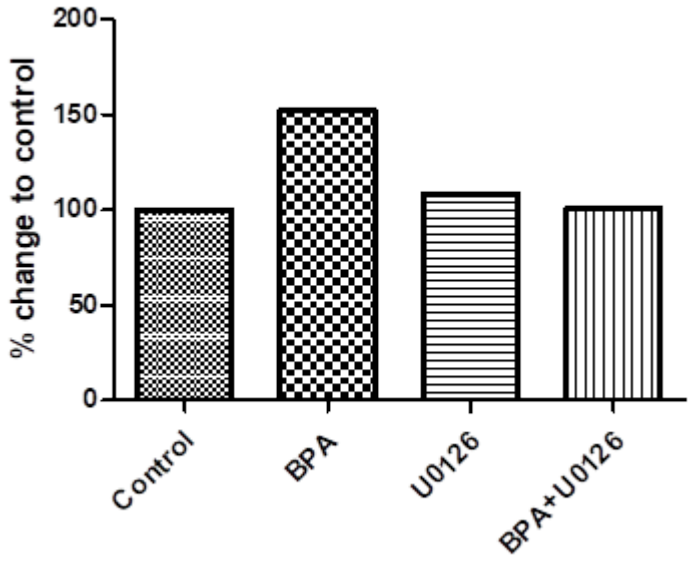

Figure 4.18 BeWo cells were treated with 3 nM BPA in the presence of MAPK-inhibitor U0126, 3 nM BPA, or a combination of both for 24 hours. There was no significant decrease in cell proliferation when cells were treated with U0126 or BPA + U0126 when compared to controls and treatment with only BPA.

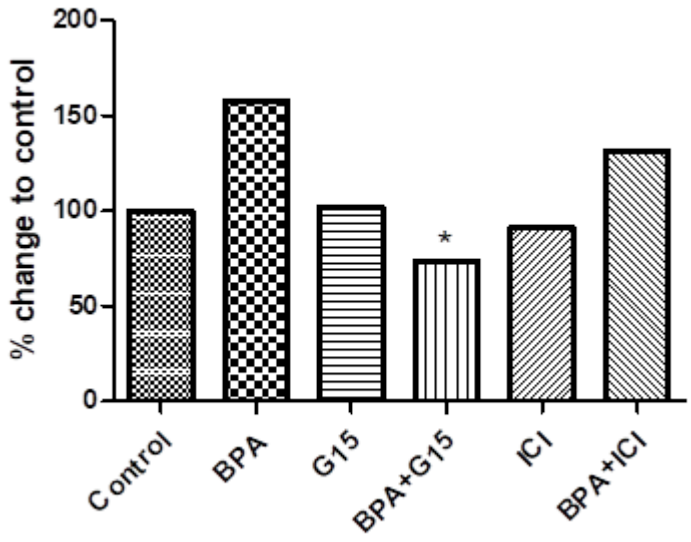

Figure 4.19 Proliferation of BeWo cells treated with 3 nM BPA and/or estrogen receptor antagonists. ICI: ERα and ERβ inhibitor, G15: GPR30 inhibitor. Proliferation compared to BPA treated cells was significantly decreased when treated with G15 ($p<0.05$).

4.2.3 Regulation of placental estrogen receptors following treatment with BPA

Next, we assessed the effects of BPA on the expression of ERs in non-syncytialised and syncytialised BeWo cells using 3 nM BPA and 10 nM BPA. In non-syncytialised BeWo cells (Figure 4.20), there appears to be an upregulaion of all three ERs after 24 hour treatment with 3 nM BPA. There was a significant upregulation of ERα ($p<0.001$) and ERβ ($p<0.05$). In syncytialised BeWo cells (Figure 4.21) there also appeared to be an upregulation of all three receptors after 24 hour 3 nM BPA treatment, however this was not significant.

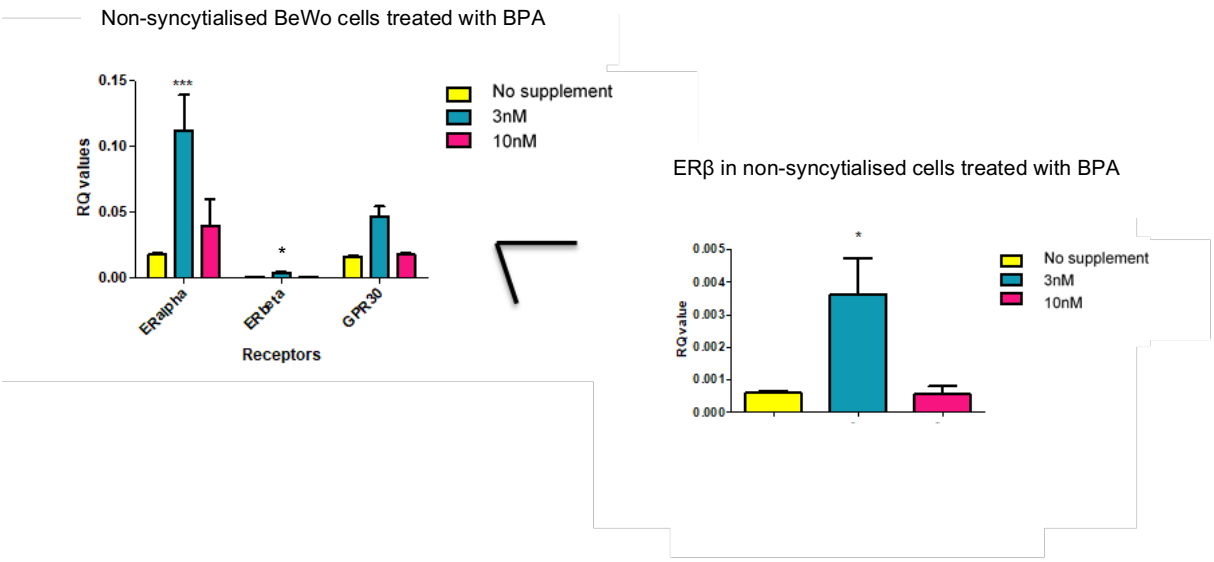

Figure 4.20 Relative levels of estrogen receptor gene expression in non-syncytialised BeWo cells. When treated with low levels (3 nM) of BPA for 24 hours, there was a highly significant increase in ERα expression in non-syncytialised BeWo cells ($p<0.001$). There was also a significant increase in ERβ when non-syncytialised BeWo cells were treated with low levels (3 nM) of BPA ($p=0.0243$). There was also an increase in GPR30 gene expression, however this was not significant.

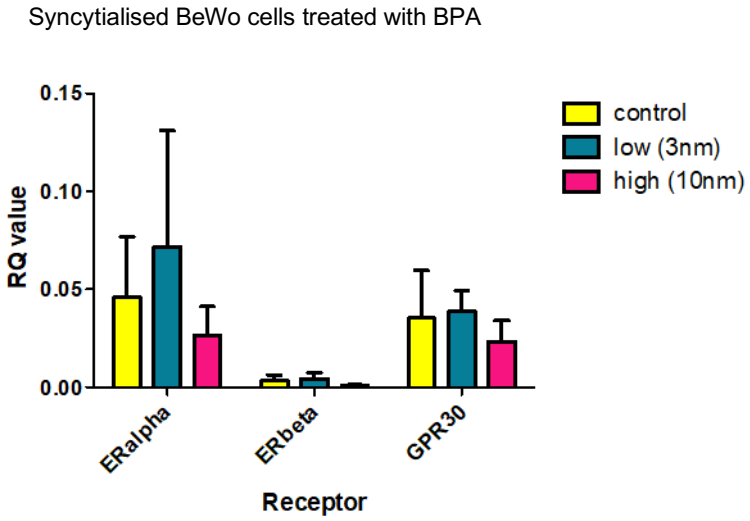

Figure 4.21 Relative levels of estrogen receptor gene expression in syncytialised BeWo cells. When cells were treated with low levels of BPA (3 nM and 10 nM) there was no significant increase or decrease in estrogen receptor expression levels.

4.3. Discussion

In this chapter we have investigated the effects of exposure to BPA on the BeWo cell line in terms of estrogen receptor regulation, mechanisms of action of BPA regarding receptor binding and intracellular signaling, as well as activation of key kinases. In doing so, we have shed light on the subject of BPA exposure and its effects on the human placenta using the BeWo cell line as an in vitro model.

Our data show that ERα gene expression is significantly upregulated in non-syncytialised BeWo cells. It is therefore possible that BPA can directly upregulate ERα. Studies have shown that E2 has the capacity to upregulate or downregulate gene expression of the ERα receptor, depending on the type of tissue, cell and conditions (Castles et al., 1997). For example, estradiol has been shown to downregulate the estrogen receptor in MCF7 breast cancer cell lines (Saceda et al., 1988). Another study showed that ERα, but not ERβ expression was upregulated in male Bombina orientalis (Boulenger) liver cells (Park & Gye, 2014). To date, there is no data regarding how activation of ERα by BPA regulates its own gene expression at placental level. Upregulation of ERα could lead to a potentiating effect of BPA, as well as a stronger response towards E2 and other activators of the estrogen receptor. This could lead to a stronger response to BPA, even at low levels. BPA can be a weak estrogen, being 10,000-100,000 times less potent than E2, or can be equally potent, eliciting cellular changes at a level of as low as 1 pM (Snyder et al., 2000; Welshons, Nagel, & Vom Saal, 2006; Welshons et al., 2003; Wozniak, Bulayeva, & Watson, 2005). The biphasic effect of BPA, meaning that lower doses of BPA have different effects than higher doses, rather than higher doses potentiating the effect, has been shown in different tissues in rats in *vivo*, as well as in the BeWo cell line (Chen et al., 2017; Jeong et al., 2017; Takai et al., 2000; Z.-Y. Wang et al., 2015). As we have shown that 3 nM BPA has led to a significant upregulation of ERα gene expression which was absent after treatment with 10 nM BPA, this could be one mechanism that would explain why lower levels could have different effects than higher levels of BPA. These effects could be specific to BeWo cells, as different cell types have been shown to have varied effects on proliferation, after exposure to BPA.

In our studies of proliferation of the BeWo cell line after BPA treatment, we found that low levels of BPA (3 nM) significantly increase BeWo cell proliferation, which is in line with in *vivo* studies in mice and rats, cultured rat epithelial prostate cells, and ovarian cell lines showing that low levels of BPA increased cell proliferation via different mechanisms (García-Arévalo et al., 2016; Huang et al., 2018; Huang, Wu, Su, Yan, & Sun, 2017; Jeong et al., 2017; Sheng, Huang, Liu, & Zhu, 2013; Shi et al., 2017). For example, proliferation of ovarian cancer cell line OVCA3 was significantly increased in cells treated with 10^{-9} Mol/L BPA for 24 hours and not when treated with 10^{-7} Mol/L BPA (Shi et al., 2017) and proliferation of rat prostate epithelial cells in culture was significantly increased after treatment with 0.1 and 1 nM BPA, as opposed to showing decreased proliferation after treatment with 10-1000nM BPA (Huang et al., 2017). In BeWo cells, higher levels of BPA (10 nM) did not have a significant effect on cell proliferation, highlighting once again the fact that lower levels of BPA have different effects on cells than higher levels do. As we have seen this pattern with ERα upregulation as well, it is possible that the proliferation of BeWo cells after 3 nM BPA treatment is an effect likely mediated via binding of BPA to ERα, although it could also be mediated by GPR30.To further investigate this hypothesis, and whether BPA acts via other mechanisms in BeWo cells, i.e. the membrane-bound receptor GPR30, further studies were conducted to investigate proliferation of BeWo cells in the presence of estrogen receptor antagonists and/or BPA. It is evident that G15 inhibited the effect of BPA, whereas cell proliferation was partially inhibited by ICI. This points to a proliferative effect of 3 nM BPA in BeWo cells involving both GPR30 and ERα or ERβ.

It is known that E2 promotes growth in different cells via both genomic receptors, through genomic mechanisms such as an increase in insulin-like growth factor (IGF) (Kumar et al., 2018; Oesterreich et al., 2001; Yashwanth, Rama, Anbalagan, & Rao, 2006), and so it is feasible that BPA would also exert this effect. Proliferation was also decreased with G15 treatment when compared to BPA treatment, implying that the proliferative effect of BPA is partly mediated via the membrane bound GPR30 estrogen receptor.

To elucidate intracellular signaling cascades influenced by the binding of BPA to estrogen receptors and whether these mechanisms play a role in BeWo cell proliferation through BPA, BeWo cells were treated with 3 nM BPA, 3 nM BPA + LY294002, BPA + U0126 and both inhibitors separately. These targets were investigated due to their role in estrogen signaling and therefore a similar effect of BPA was postulated. In our studies of proliferation, we found a significant decrease of BeWo cell proliferation after inhibition of AKT, in the presence or absence of 3 nM BPA. These results point to a non-genomic proliferative effect of BPA on BeWo cells that is mediated through some way via a signaling pathway that is regulated by AKT. One way this could be achieved is via the AKT/PTEN pathway, which has been shown to respond to estrogen as a stimulus with increased proliferation in different types of tissues, such as primordial germ cells in *vitro* (Moe-Behrens et al., 2003). Another pathway involving AKT which results in an increase of proliferation due to estrogen is the PI3K/AKT pathway, which stimulates proliferation in breast cancer cells via ERα (Lee et al., 2005). Studies have shown that non-genomic effects of estrogen can be achieved by either the membrane bound G-protein-coupled receptor GPR30 or membrane bound classical estrogen receptors (Kumar et al., 2018) and that estrogen has a proliferative effect on certain cancer cells via non-genomic intracellular signaling mediated by ERα stimulation, one of these pathways being the P13K/AKT pathway, as well as pathways involving ERK signaling (Acconcia & Marino, 2011).
Furthermore, BPA specifically has been shown to have varying effects on intracellular target phosphorylation, depending on the type of cell. Both p-AKT and p-ERK1/2 have been shown to be upregulated by BPA in rat mammary gland (Betancourt, Mobley, Russo, & Lamartiniere, 2010; Lamartiniere, Jenkins, Betancourt, Wang, & Russo, 2011) as well as AKT1 being upregulated in human breast epithelial cells after low levels of BPA treatment (Goodson et al., 2011). On the other hand, p-AKT has been shown to be downregulated after BPA treatment in rat sertoli cells (Wang et al., 2015) as well as rat hippocampi (Wang et al., 2016). As BPA has shown contradictory effects on regulation of intracellular targets, information about the regulation of these targets in BeWo cells was needed.

Our data has shown an effect of BPA on the proliferation of BeWo cells. We have also explored the mechanisms behind the proliferation of BeWo cells after BPA treatment

and have been able to determine key targets as well as receptors by which BPA likely mediates its action. Taken together, our data suggests that proliferative BPA function in BeWo cells is most likely mediated by ERα, as we have shown that this receptor is upregulated by 3 nM concentrations of BPA, and also that the inhibition of ERα in combination with BPA clearly resulted in a loss of proliferation in BeWo cells, which was less than when cells were treated with the antagonist alone. Furthermore, we have seen that the inhibition of AKT via LY294002 significantly decreased proliferation in BeWo cells with and without 3 nM BPA treatment. As we have seen an increase in AKT phosphorylation on a protein level after treatment with 10 nM BPA for 60 mins, this further suggests that this intracellular target plays a role in BPA signaling. Given that AKT signaling plays a major role in effects mediated by estrogen, and that ERα has often been implicated in triggering AKT signaling, for example in endometrial cancer cells (Haque et al., 2018), human breast cancer, and human kidney cells (Das, Datta, Chatterjee, & Ghosh, 2016) it is plausible that BPA, as a xenoestrogen, would exert these effects in BeWo cells.

Chapter 5

Use of non-biased gene microarray analyses to assess the effects of BPA *in vitro*

5.1. Introduction

In order to assess the effects of BPA on the placenta, it is essential to examine how BPA affects the whole genome via a non-biased screen. This way, an overview of main genes and pathways affected by BPA is presented and it is possible to examine which aspects of the cell, be it proliferation, invasion, or differentiation, to name just a few, are primarily altered. In this chapter, we assessed the effects BPA has on the BeWo placental cell lines in both non-syncytialised and syncytialised states, using a gene microarray. In order to elucidate which genes and pathways were most affected by the compound, the most significantly upregulated and downregulated genes after BPA treatment were found, and, using bioinformatics analysis, the most differentially regulated pathways analysed.

To our knowledge, microarray analysis has not been performed on BeWo placental cell lines treated with BPA to date. In fact, there are only a handful of studies that use microarray analysis when using the BeWo cell line as a placental model. As there is still a paucity of data regarding the effects of BPA on the placenta, it seemed vital to use this method in order to gain understanding of how human pregnancies might be affected by this compound. Studies have shown a link between a multitude of pathologies in pregnancy and BPA levels, but information on whether these pathologies are caused by effects of BPA on the human placenta, or what processes within the cell are disrupted, is lacking.

Although the effects of BPA have not extensively been evaluated using the BeWo cell line, some studies have outlined various effects of BPA on other placental models, such as the HTR-8/SVneo cell line (Spagnolotti et al, 2015; Lan et al, 2017), JEG-3 cell line (Pérez-Albaladejo et al, 2017) villous explant cultures (Lan et al, 2017), animal models (Tait et al, 2015; Lee et al, 2016; Lan et al, 2017) and term placental explants (Rajakumar et al, 2015; Xu et al, 2015; Sieppi et al, 2016). Although these studies

have added valuable information to the literature, it is necessary to study the whole genome with a non-biased screen in order to get a fuller picture of the effects of BPA on the placenta.

In order to assess genes, cell functions and pathways affected by the treatment of syncytialised and non-syncytialised BeWo cells with BPA (3 nM, 10 nM), cells were treated with BPA for 24 hours using starved mediaCells were then lysed and RNA was extracted. After RNA quality screening, RNA was used for microarray analysis to asses up- or downregulation of gene expression in the entire genome after BPA treatment. Read-outs were assessed by sorting genes from highest to lowest p-value and highest to lowest fold change and analysing differentially expressed genes using the bioinformatics analysis software FunRich. This programme performs functional enrichment as well as gene and protein interaction network analysis. FunRich analyses biological processes, cellular components, protein domains and molecular functions, expression sites, biological pathways, transcription factors and provides a clinical synopsis of phenotypic terms. In order to further analyse data, differentially expressed gene lists were uploaded to the online bioinformatics application Enrichr (Chen et al., 2013). This platform uses a vast array of different genetic databases in order to establish differentially regulated signaling pathways, transcription factors, and diseases, among other analyses. Read-outs of the two different bioinformatic platforms were compared in order to assess reliability.

The main aim of this chapter is to assess gene expression changes in BeWo cells as well as human placental explants after treatment with 3 nM and 10 nM BPA for 24 hours and to analyse the outputs in the context of bioinformatic analysis and thereby identify common pathways and diseases affected by BPA, as well as identifying relevant upregulated genes. Therefore, this chapter has been structured into 3 main aims.

1. Determine differentially expressed genes in non-syncytialised BeWo cells treated with 3 nM and 10 nM BPA for 24 hours using microarray and perform bioinformatic analysis on outputs. Validate significantly upregulated genes of interest with qPCR and immunostaining.

2. Determine differentially expressed genes in syncytialised BeWo cells treated with 3 nM and 10 nM BPA for 24 hours using microarray and perform bioinformatic analysis on outputs. Validate significantly upregulated genes of interest with immunostaining.

3. Determine differentially expressed genes in placental explants from term placenta treated with 3 nM and 10 nM BPA for 24 hours using microarray and perform bioinformatic analysis on outputs. Assess overlap between explants and BeWo cells.

5.2 Results

5.2.1. Bioinformatic analysis of BeWo microarray results

Outputs from microarray analysis were analysed using the bioinformatics gene enrichment tool Funrich. Significantly up- and downregulated genes were uploaded to the software in order to assess biological processes, cellular components, protein domains and molecular functions, expression sites, biological pathways, transcription factors and clinical phenotypes. Outputs from microarray were also analysed via the gene enrichment tool Enrichr, which uses different databases and shows all results from different databases. Furthermore, overlap of differentially regulated genes between groups was analysed using Venn diagrams created with the Funrich tool.

5.2.1.1 Bioinformatic analysis of non-syncytialised BeWo cells treated with BPA

A Venn diagram showing differentially expressed gene overlap in non-syncytialised 3 nM and 10 nM treated BeWo cells (figure 5.1) showed that 194 genes were differentially expressed in both cells (gene names shown in appendix, table 1.) The Venn diagram also shows a large difference between the two sets, with 1195 genes being differentially regulated in 3 nM treated BeWo cells, whereas to nM treated BeWo cells only showed differential regulation in 477 genes.

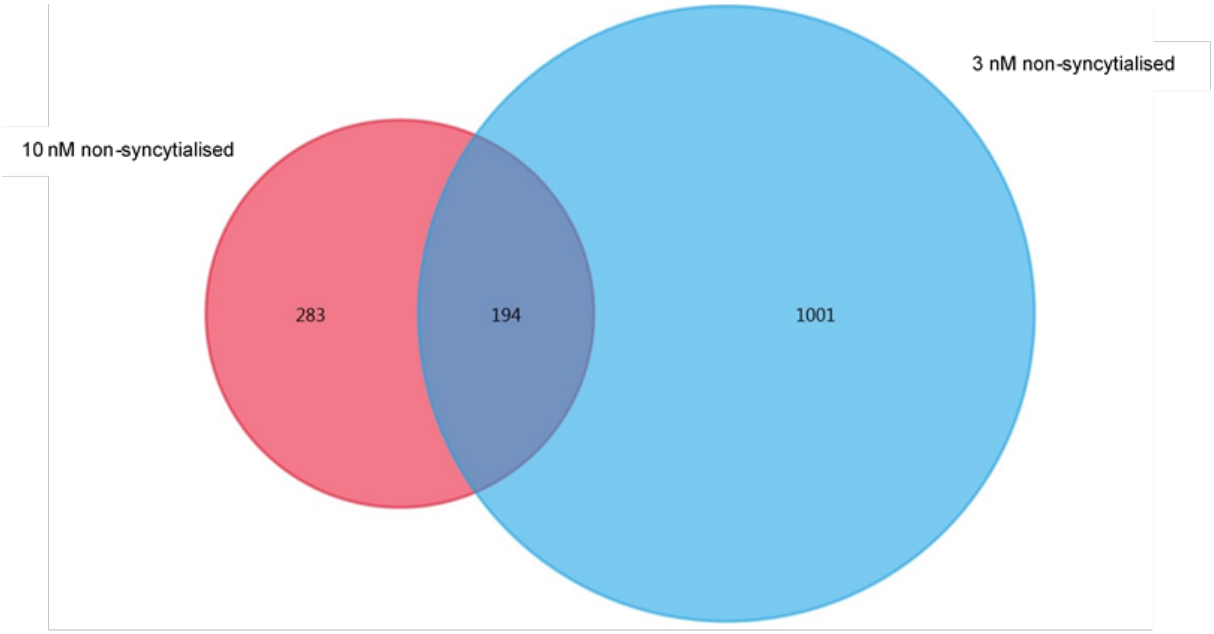

Figure 5.1 Venn diagram showing differentially expressed gene overlap between 10 nM non-syncytialised and 3 nM non-syncytialised BeWo cells. In total, 194 genes were differentially expressed in both gene sets. The total set of differentially expressed genes was much larger in 3 nM non-syncytialised BeWo cells with a total number of 1195 genes, compared to 477 genes. Shared differentially upregulated genes: see Appendix Table 1.

Top upregulated genes in 3 nM treated non-syncytialised cells are shown below (table 5.1). They have been organised by highest to lowest significance (p-value) and their functions are described. The most significantly upregulated gene in this set is cytoplasmatic polyadenylation element-binding protein 1 (CPEB1). This protein is vital for cell cycle progression, particularly prophase entry.

GENE	FUNCTION	P-VALUE
Cytoplasmic polyadenylation element-binding protein 1 (CPEB1)	Sequence-specific RNA-binding protein regulating mRNA cytoplasmic polyadenylation and initiation of translation. Cell cycle progression, particularly prophase entry.	2.6×10^{-4}
Rap guanine nucleotide exchange factor 1 (RAPGEF1)	Guanine nucleotide-releasing protein that transduces signals from CRK to activate RAS. involved in establishment of basal endothelial barrier function.	5.56×10^{-4}
Myosin light chain 3 (MYL3)	Regulatory light chain of the muscular protein myosin that does not bind calcium.	9.21×10^{-4}
Caveolin-1 (CAV1)	Possible function as a scaffolding protein in caveolae. Can regulate G-protein alpha subunits and their activity. Recruits proteins to caveolar membranes.	0.001
Calsyntenin-3 (CLSTN3)	Possible role in calcium-mediated postsynaptic signals. Plays a role in APP metabolism and maturation.	0.002
Hydroxycarboxylic acid receptor 3 (HCAR3)	G-protein coupled receptor involved in regulation of adipocyte lipolysis as negative feedback to counteract prolipolytic influences. Receptor for 3-OH-octanoid acid.	0.002
Serpin B9 (SERPINB9)	Inhibitor of Granzyme B, anti-apoptotic, response to estrogen stimulus	0.003
Alanine--glyoxylate aminotransferase 2 (AGXT2)	Metabolises asymmetric dimethylarginine (ADMA) to dimethylguanidino valeric acid (DMGV) thereby increasing NOS activity leading to vascular dilation.	0.003
Transmembrane protein 45B (TMEM45B)	Promotes proliferation and inhibits apoptosis in pancreatic cancer cells (Zhao et al., 2016), promotes proliferation in gastric cancer cells (Shen, Yu, Yu, Liu, & Cui, 2018), upregulated in human lung cancer cells (R. Hu et al., 2016), promotes proliferation in osteosarcoma cells (Y. Li et al., 2017)	0.003
Eukaryotic translation initiation factor 4E type 2 (EIF4E2)	Repressor of initiation of translation by binding to mRNA cap	0.003

Table 5.1. Top 10 upregulated genes in 3 nM BPA treated non-syncytialised BeWo cells including protein functions and p-values of upregulation. Where not cited, functions have been summarised from the UniProt protein database (https://www.uniprot.org). The most significantly upregulated gene is CPEB1, followed by RAPGEF1, MYL3, CAV1, CLSTN3, HCAR3, SERPINB9, AGXT2, TMEM45B and EIF4E2, respectively.

Top upregulated genes in 10 nM treated non-syncytialised cells are shown below (table 5.2). They have been organised by highest to lowest significance (p-value) and their functions are described. The most significantly upregulated gene in this set is caveolin-1 (CAV1). This protein is a scaffolding protein in membrane caveolae, as well as being able to regulate g-protein alpha subunits and recruiting other proteins to caveolar membranes.

GENE	FUNCTION	P-VALUE
Caveolin-1 (CAV1)	Possible function as a scaffolding protein in caveolae. Can regulate G-protein alpha subunits and their activity. Recruits proteins to caveolar membranes.	4.66×10^{-4}
Myosin light chain 3 (MYL3)	Regulatory light chain of the muscular protein myosin that does not bind calcium.	0.004
Cerebellin-1 (CBLN1)	Required for development of the nervous system; synaptic plasticity	0.005
Ankyrin-3 ANK3	Links membrane and cytoskeleton as well as regulating cell adhesion molecules and renal Mg^{2+} absorption	0.007
Thiopurine S-methyltransferase (TPMT)	Modulates thiopurine drug toxicity	0.009
Leptin (LEP)	Regulation of energy homeostasis and body weight control. Appetite modulating, pro-angiogenic, inhibitor of glucose absorption in the bowel, pro-inflammatory	0.01
Hyaluronan and proteoglycan link protein 3 (HAPLN3)	Hyaluronic acid binding	0.01
Sperm flagellar 1 (SPEF1)	Microtubule associated protein that promotes establishment of axis of microtubules	0.01
Placenta-specific 8 (PLAC8)	Enriched protein in the placenta, promotes cell proliferation and tumour progression (W.-L. Chang et al., 2018; Y. Jia et al., 2018; Kolluru et al., 2017; C. Li et al., 2014; R. Yang et al., 2018; Zou et al., 2016)	0.01
Eukaryotic translation initiation factor 4E type 2 (EIF4E2)	Repressor of initiation of translation by binding to mRNA cap	0.01

Table 5.2. Top 10 upregulated genes in 10 nM BPA treated non-syncytialised BeWo cells including protein functions and p-values of upregulation. Where not cited, functions have been summarised from the UniProt protein database (https://www.uniprot.org). The most significantly upregulated gene is CAV1, followed by MYL3, CBLN1, ANK3, TPMT, LEP, HAPLN3, SPEF1, PLAC8, and EIF4E2, respectively.

Next, gene enrichment analysis in non-syncytialised BeWo cells treated with 3 nM and 10 nM BPA was performed using the FunRich and Enrichr databases. Gene sets were analysed in terms of their common pathways, molecular functions, common gene

transcription factors and clinical phenotypes. Tables and graphs showing most significant gene enrichment are shown below. Figure 5.2 shows enriched molecular functions of genes from BeWo cells treated with 3 nM BPA. Using this database, it was shown that guanyl-nucleotide exchange factor (GEF) activity was significantly enriched (p=0.049). Using the database Enrichr (Table 5.3) showed that the top 10 significantly regulated molecular functions also included Rac GEF (p=0.00009) and Rho GEF (p=0.0002) activity, among others, showing that both platforms yielded similar results.

Next, we investigated biological pathway gene enrichment in 3 nM treated BeWo cells using both platforms. Funrich data (figure 5.3) showed that regulation of RAC1 activity and RAC1 signaling pathway were significantly enriched (p=0.007), and that regulation of RhoA activity and RhoA signaling were significantly enriched (p=0.007). Enrichr data using the NCI Nature database 2016 for pathway gene enrichment (table 5.4) showed that the top significant differentially regulated biological pathway was “insulin resistance” (p=0.01). Using the KEGG 2016 database via Enrichr for biological pathways (table 5.5), the most significantly regulated pathway was regulation of RhoA activity (p=0.0003) and the 9th most significantly regulated pathway was the RhoA signaling pathway (p=0.05). The WikiPathways 2016 database via Enrichr (table 5.6) showed an upregulation of Leptin Insulin Overlap pathways (p=0.0014), differentiation of white and brown adipose tissue pathways (p=0.015), insulin signaling pathway (p=0.03), and leptin signaling pathway (p=0.034).

When looking at gene enrichment and transcription factors, when using the FunRich database (figure 5.4), the most differentially regulated transcription factor was RREB1 (p=0.004). Using Enrichr (table 5.7), the most regulated transcription factor was ZFHX3 (p=0.001).

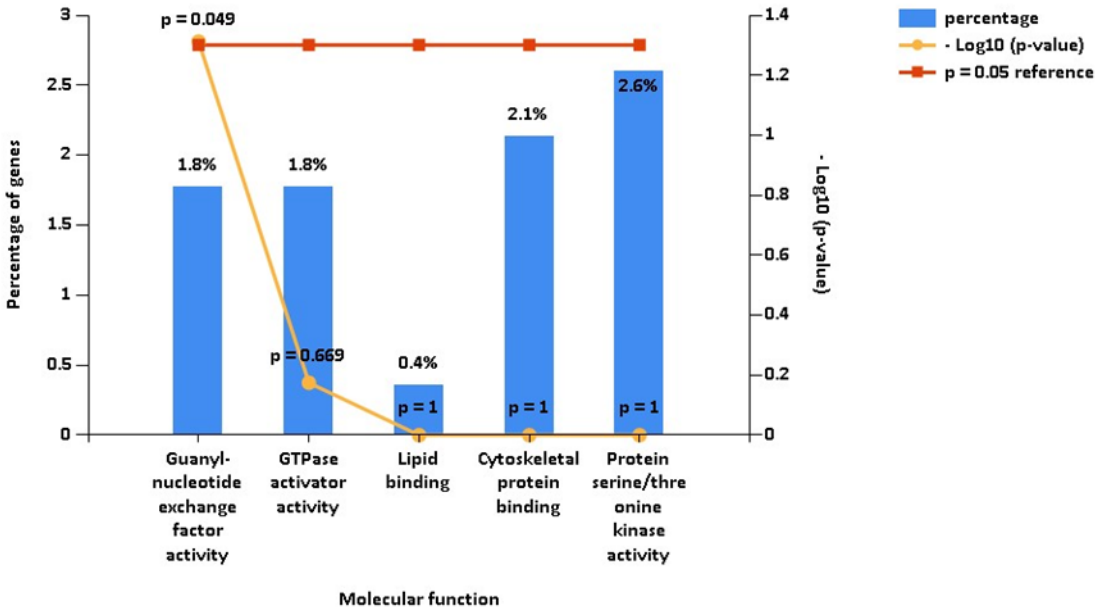

Figure 5.2 Funrich top 5 enriched molecular functions of genes from BeWo cells treated with 3 nM BPA. Guanyl-nucleotide exchange factor (GEF) activity was significantly enriched (p=0.049). Percentages show percentage of genes involved in the molecular function. The orange line indicates cut off for significance (p<0.05) and the yellow line indicates -log10 p-values.

Index	Name	P-value
1	Rac guanyl-nucleotide exchange factor activity (GO:0030676)	0.00009435
2	Rho guanyl-nucleotide exchange factor activity (GO:0005089)	0.0002070
3	intermediate filament binding (GO:0019215)	0.003712
4	transcriptional activator activity, RNA polymerase II transcription regulatory region sequence-specific binding (GO:0001228)	0.01348
5	SUMO ligase activity (GO:0061665)	0.01979
6	RNA polymerase II distal enhancer sequence-specific DNA binding (GO:0000980)	0.02024
7	keratin filament binding (GO:1990254)	0.02445
8	arginine binding (GO:0034618)	0.02445
9	ubiquitin protein ligase activity (GO:0061630)	0.02809
10	RNA polymerase II transcriptional repressor activity, metal ion regulated core promoter proximal region sequence-specific binding (GO:0001214)	0.03964

Table 5.3 Top 10 significant GO-terms of molecular function associated with differentially expressed genes after 3 nM BPA treatment of BeWo cells using GO molecular function 2017b database via Enrichr. Terms are ordered from most significant to least significant. Rac GEF exchange factor activity is the most significantly regulated GO-term.

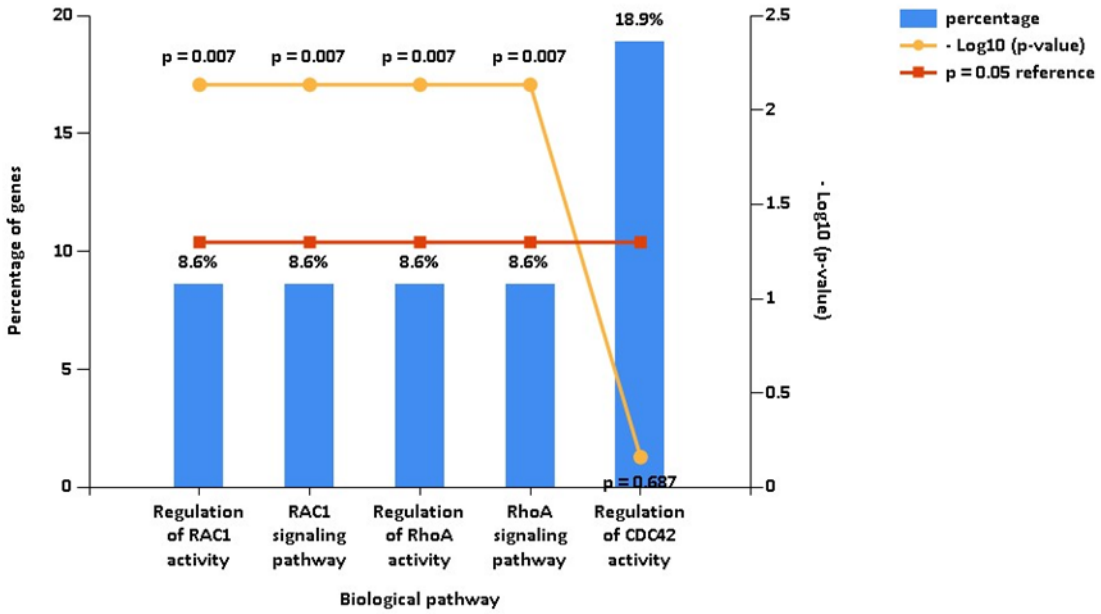

Figure 5.3 Funrich top 5 enriched biological pathways of genes from BeWo cells treated with 3 nM BPA. Regulation of RAC1 activity and RAC1 signaling pathway were significantly enriched (p=0.007), regulation of RhoA activity and RhoA signaling pathway were also significantly enriched (p=0.007).

Index	Name	P-value
1	Insulin resistance_Homo sapiens_hsa04931	0.01312
2	Focal adhesion_Homo sapiens_hsa04510	0.05880
3	Platelet activation_Homo sapiens_hsa04611	0.06095
4	Hypertrophic cardiomyopathy (HCM)_Homo sapiens_hsa05410	0.05914
5	Type II diabetes mellitus_Homo sapiens_hsa04930	0.06492
6	Estrogen signaling pathway_Homo sapiens_hsa04915	0.07147
7	Glycosphingolipid biosynthesis - globo series_Homo sapiens_hsa00603	0.04722
8	Insulin signaling pathway_Homo sapiens_hsa04910	0.07262
9	Endocytosis_Homo sapiens_hsa04144	0.09626
10	Dilated cardiomyopathy_Homo sapiens_hsa05414	0.08857

Table 5.4 Top 10 terms of biological pathways associated with differentially expressed genes after 3 nM BPA treatment of BeWo cells using NCI Nature 2016 database via Enrichr. The most significantly regulated pathway is insulin resistance (p=0.013).

Index	Name	P-value
1	Regulation of RhoA activity_Homo sapiens_49ece019-6195-11e5-8ac5-06603eb7f303	0.0003159
2	a6b1 and a6b4 Integrin signaling_Homo sapiens_73d1a893-6186-11e5-8ac5-06603eb7f303	0.01654
3	ATF-2 transcription factor network_Homo sapiens_c76c900a-618a-11e5-8ac5-06603eb7f303	0.02133
4	ErbB4 signaling events_Homo sapiens_6104ebb2-6192-11e5-8ac5-06603eb7f303	0.02402
5	Reelin signaling pathway_Homo sapiens_054f432f-6195-11e5-8ac5-06603eb7f303	0.02707
6	Nephrin/Neph1 signaling in the kidney podocyte_Homo sapiens_6cfb9873-6194-11e5-8ac5-06603eb7f303	0.03513
7	IL2-mediated signaling events_Homo sapiens_a2a1883c-6193-11e5-8ac5-06603eb7f303	0.04441
8	EPO signaling pathway_Homo sapiens_20fe3c0e-6192-11e5-8ac5-06603eb7f303	0.04453
9	RhoA signaling pathway_Homo sapiens_5c6b5f5c-6195-11e5-8ac5-06603eb7f303	0.04999
10	Beta5 beta6 beta7 and beta8 integrin cell surface interactions_Homo sapiens_108637d6-618e-11e5-8ac5-06603eb7f303	0.07733

Table 5.5 Top 10 terms of biological pathways associated with differentially expressed genes after 3 nM BPA treatment of BeWo cells using KEGG 2016 database via Enrichr. The most significantly regulated pathway is regulation of RhoA activity (p=0.0003). The 9th most regulated pathway is the RhoA signaling pathway (p=0.05).

Index	Name	P-value
1	Leptin Insulin Overlap_Mus musculus_WP578	0.001369
2	Differentiation of white and brown adipocyte _Homo sapiens_WP2895	0.01474
3	Kit receptor signaling pathway_Homo sapiens_WP304	0.02343
4	IL-3 Signaling Pathway_Mus musculus_WP373	0.02988
5	Insulin Signaling_Homo sapiens_WP481	0.03006
6	Leptin signaling pathway_Homo sapiens_WP2034	0.03426
7	IL-2 Signaling Pathway_Homo sapiens_WP49	0.03737
8	Integrin-mediated Cell Adhesion_Mus musculus_WP6	0.03405
9	Alpha6-Beta4 Integrin Signaling Pathway_Mus musculus_WP488	0.03610
10	Insulin Signaling_Mus musculus_WP65	0.04249

Table 5.6 Top 10 significant GO-terms of biological pathways associated with differentially expressed genes after 3 nM BPA treatment of BeWo cells using WikiPathways 2016 database via Enrichr. Leptin Insulin Overlap (p=0.001), differentiation of white and brown adipose tissue (p=0.015), insulin signaling (p=0.03), and leptin signaling pathway (p=0.034) were all significantly regulated.

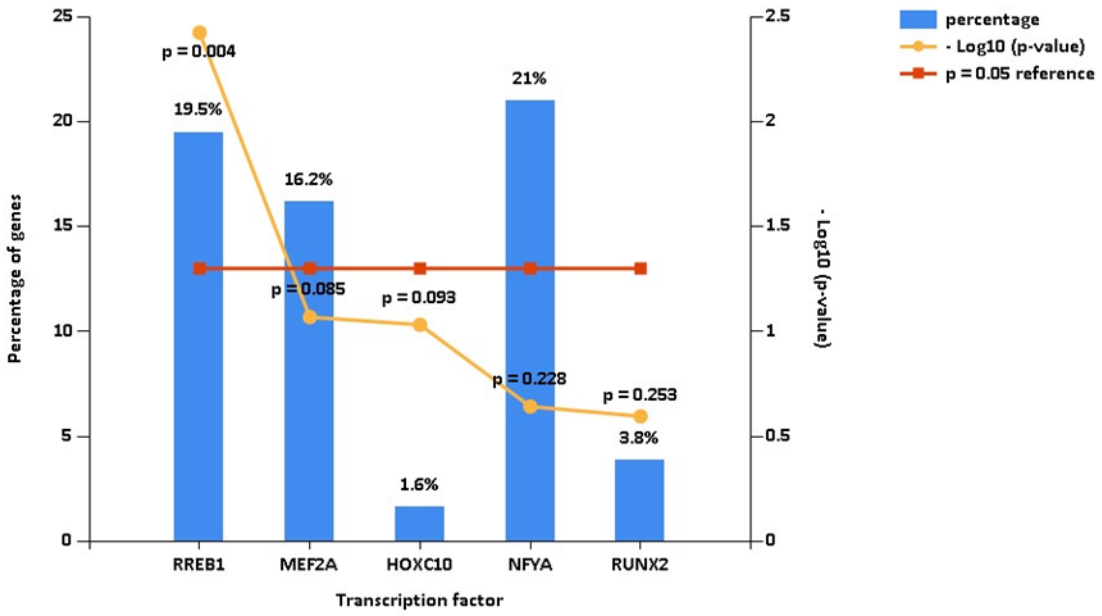

Figure 5.4 Funrich enriched transcription factors of genes from BeWo cells treated with 3 nM BPA. RREB1 transcription factor is significantly enriched (p=0.004).

Index	Name	P-value
1	ZFHX3_human_tf_ARCHS4_coexpression	0.001032
2	ZNF638_human_tf_ARCHS4_coexpression	0.002029
3	AFF1_human_tf_ARCHS4_coexpression	0.002029
4	LCORL_human_tf_ARCHS4_coexpression	0.003856
5	ZNF91_human_tf_ARCHS4_coexpression	0.003856
6	TFCP2L1_human_tf_ARCHS4_coexpression	0.003856
7	FOSB_human_tf_ARCHS4_coexpression	0.003856
8	ZNF704_human_tf_ARCHS4_coexpression	0.003856
9	NFAT5_human_tf_ARCHS4_coexpression	0.007083
10	RREB1_human_tf_ARCHS4_coexpression	0.007083

Table 5.7. Top 10 significant transcription factors associated with differentially expressed genes after 3 nM BPA treatment of BeWo cells using ARCHS4 TFs Coexp database via Enrichr.

In non-syncytialised BeWo cells treated with 10 nM BPA, the Funrich database did not show any significantly differentially expressed molecular functions. Using the GO molecular function 2017b database via Enrichr, Rac guanyl nucleotide exchange factor activity was differentially expressed (p=0.01). When looking at differentially expressed biological processes, there was no significant change when analysing the data with Funrich, although there was a high number of differentially expressed apoptotic processes when using the GO biological processes database via Enrichr, including positive regulation of apoptotic processes in development (p=0.002). When looking at biological pathways, there were no significantly enriched pathways when using the Funrich platform, the KEGG 2016 database via Enrichr however showed significant enrichment of the insulin resistance pathway (p=0.005), among other significantly regulated pathways. Via Enrichr, the Wikipathways 2016 database for biological pathways showed a significant regulation of differentiation of white and brown adipose tissues pathways (p=0.003) and using the NCI Nature 2016 database for biological pathways via Enrichr, regulation of RAC1 activity was significantly regulated (p=0.012).

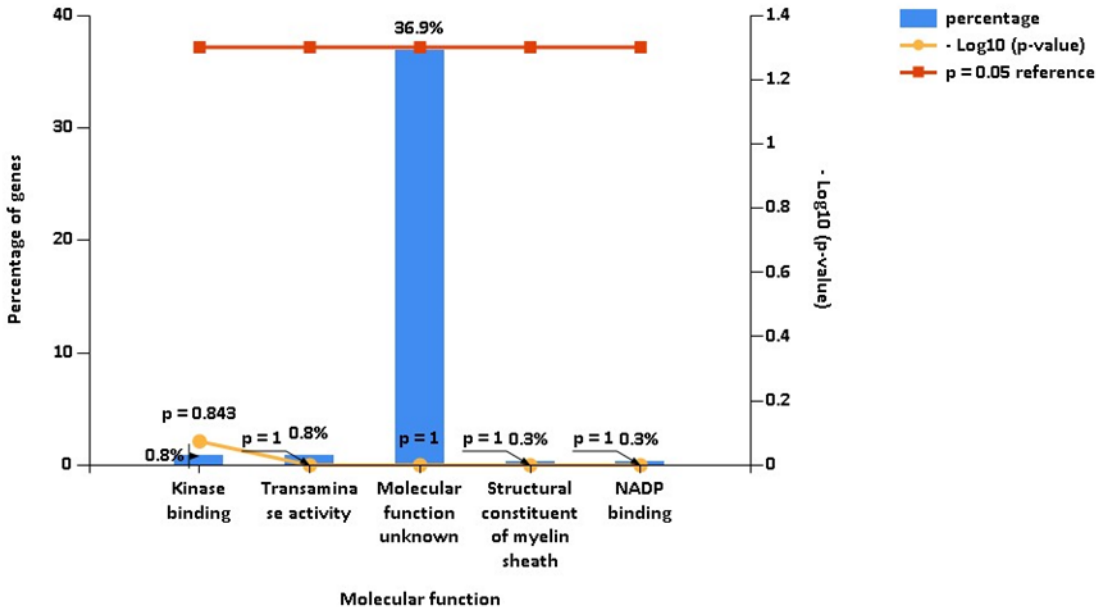

Figure 5.5. Funrich enriched molecular function of genes from BeWo cells treated with 10 nM BPA.

Index	Name	P-value
1	enhancer sequence-specific DNA binding (GO:0001158)	0.0002082
2	polymerase III regulatory region sequence-specific DNA binding (GO:0000992)	0.0008361
3	mitochondrial RNA polymerase regulatory region sequence-specific DNA binding (GO:0001044)	0.0007441
4	bacterial-type RNA polymerase regulatory region sequence-specific DNA binding (GO:0000984)	0.0007441
5	transcription termination site sequence-specific DNA binding (GO:0001147)	0.0009365
6	core promoter proximal region sequence-specific DNA binding (GO:0000987)	0.002529
7	Rac guanyl-nucleotide exchange factor activity (GO:0030676)	0.01064
8	transcription regulatory region sequence-specific DNA binding (GO:0000976)	0.03124
9	acetyl-CoA:CoA antiporter activity (GO:0015325)	0.01029
10	ubiquitin protein ligase activity (GO:0061630)	0.04821

Table 5.8. Top 10 significant molecular functions associated with differentially expressed genes after 10 nM BPA treatment of BeWo cells using GO molecular function 2017b database via Enrichr. Rac guanyl-nucleotide exchange factor activity is differentially expressed (p=0.01)

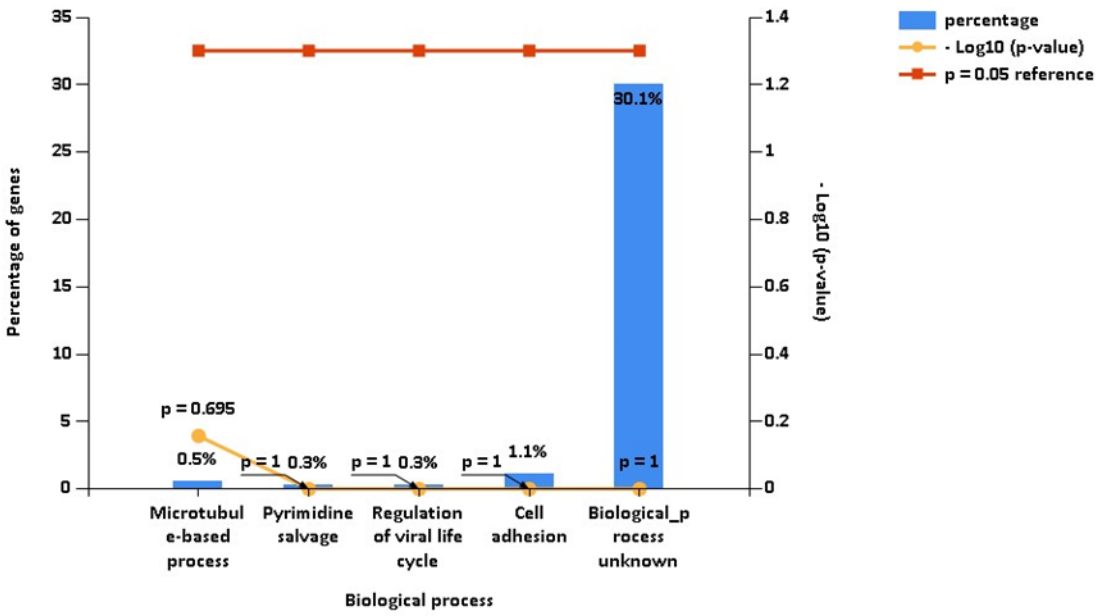

Figure 5.6. Funrich enriched biological processes of genes from BeWo cells treated with 10 nM BPA.

Index	Name	P-value
1	positive regulation of neuron apoptotic process (GO:0043525)	0.0008131
2	positive regulation of cysteine-type endopeptidase activity involved in apoptotic process (GO:0043280)	0.001415
3	positive regulation of apoptotic process involved in development (GO:1904747)	0.001543
4	positive regulation of apoptotic process in other organism (GO:0044533)	0.001543
5	positive regulation of leukocyte apoptotic process (GO:2000108)	0.001543
6	positive regulation of fat cell apoptotic process (GO:1904651)	0.001543
7	positive regulation of epithelial cell apoptotic process (GO:1904037)	0.001543
8	positive regulation of compound eye retinal cell apoptotic process (GO:1901694)	0.001543
9	positive regulation of mesenchymal cell apoptotic process (GO:2001055)	0.001543
10	positive regulation of myofibroblast cell apoptotic process (GO:1904522)	0.001543

Table 5.9. Top 10 significant biological processes associated with differentially expressed genes after 10 nM BPA treatment of BeWo cells using GO biological process 2017b database via Enrichr. There was a significantly regulated gene expression of positive regulation of apoptotic processes involved in development (p=0.002), among other differentially expressed biological processes relating to apoptosis.

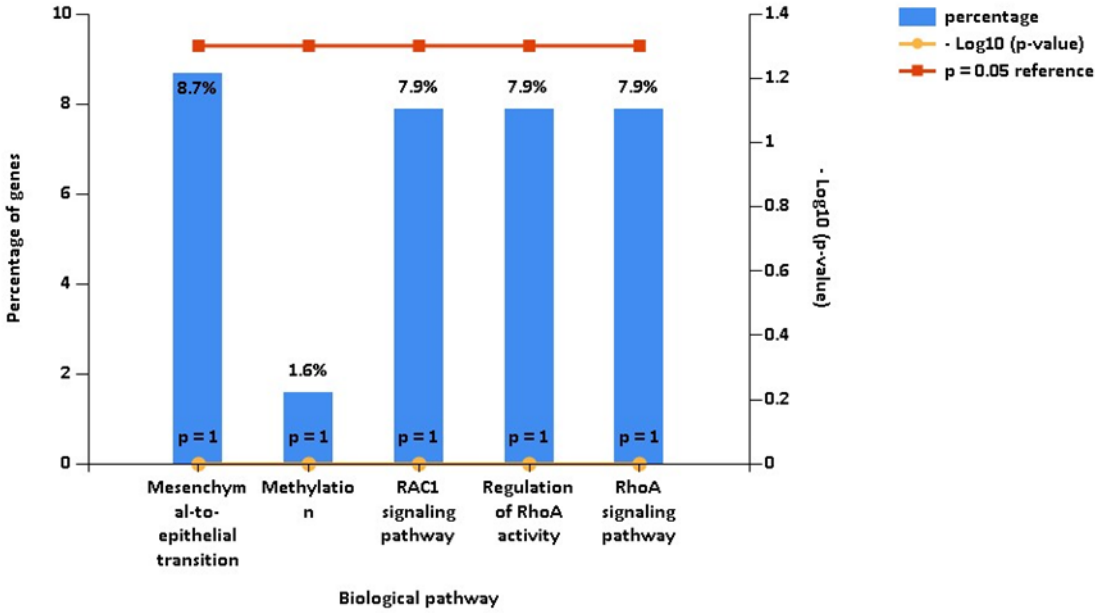

Figure 5.7. Funrich enriched biological pathways of genes from BeWo cells treated with 10 nM BPA.

Index	Name	P-value
1	Insulin resistance_Homo sapiens_hsa04931	0.004594
2	Alanine, aspartate and glutamate metabolism_Homo sapiens_hsa00250	0.009322
3	Dilated cardiomyopathy_Homo sapiens_hsa05414	0.02067
4	Dorso-ventral axis formation_Homo sapiens_hsa04320	0.02580
5	Hypertrophic cardiomyopathy (HCM)_Homo sapiens_hsa05410	0.04831
6	mTOR signaling pathway_Homo sapiens_hsa04150	0.05479
7	Proteoglycans in cancer_Homo sapiens_hsa05205	0.05506
8	Progesterone-mediated oocyte maturation_Homo sapiens_hsa04914	0.08526
9	Estrogen signaling pathway_Homo sapiens_hsa04915	0.08813
10	Hepatitis C_Homo sapiens_hsa05160	0.09907

Table 5.10. Top 10 biological pathways associated with differentially expressed genes after 10 nM BPA treatment of BeWo cells using KEGG 2016 database via Enrichr. The insulin resistance pathway is significantly differentially regulated (p=0.005).

Index	Name	P-value
1	Differentiation of white and brown adipocyte _Homo sapiens_WP2895	0.002715
2	Methylation_Mus musculus_WP1247	0.01829
3	Methylation Pathways_Homo sapiens_WP704	0.01829
4	Circadian rythm related genes_Homo sapiens_WP3594	0.05102
5	Alanine and aspartate metabolism_Homo sapiens_WP106	0.03198
6	Osteopontin Signaling_Homo sapiens_WP1434	0.03721
7	Alanine and aspartate metabolism_Mus musculus_WP240	0.03198
8	Splicing factor NOVA regulated synpatic proteins_Mus musculus_WP1983	0.07818
9	Integrin-mediated Cell Adhesion_Mus musculus_WP6	0.08813
10	Primary Focal Segmental Glomerulosclerosis FSGS_Homo sapiens_WP2572	0.09306

Table 5.11. Top 10 biological pathways associated with differentially expressed genes after 10 nM BPA treatment of BeWo cells using WikiPathways 2016 database via Enrichr. Differentiation of white and brown adipocyte pathways were siginificantly regulated (p=0.003).

Index	Name	P-value
1	Regulation of RAC1 activity_Homo sapiens_351aacd6-6195-11e5-8ac5-06603eb7f303	0.01243
2	Integrin family cell surface interactions_Homo sapiens_1ca2bf67-6194-11e5-8ac5-06603eb7f303	0.02333
3	Direct p53 effectors_Homo sapiens_67c3b75d-6191-11e5-8ac5-06603eb7f303	0.04465
4	Arf6 downstream pathway_Homo sapiens_1e972087-618a-11e5-8ac5-06603eb7f303	0.04856
5	Circadian rhythm pathway_Homo sapiens_ffb11b85-6190-11e5-8ac5-06603eb7f303	0.04856
6	Canonical Wnt signaling pathway_Homo sapiens_9dbe253d-618f-11e5-8ac5-06603eb7f303	0.08132
7	TNF receptor signaling pathway_Homo sapiens_316be05e-6196-11e5-8ac5-06603eb7f303	0.09657
8	Signaling events mediated by PRL_Homo sapiens_bb67523a-6195-11e5-8ac5-06603eb7f303	0.1034
9	Plexin-D1 Signaling_Homo sapiens_e3068f36-6194-11e5-8ac5-06603eb7f303	0.1111
10	PLK3 signaling events_Homo sapiens_eac8a4b8-6194-11e5-8ac5-06603eb7f303	0.1137

Table 5.12. Top 10 biological pathways associated with differentially expressed genes after 10 nM BPA treatment of BeWo cells using NCI-Nature 2016 database via Enrichr. Regulation of RAC1activity is significantly regulated (p=0.012).

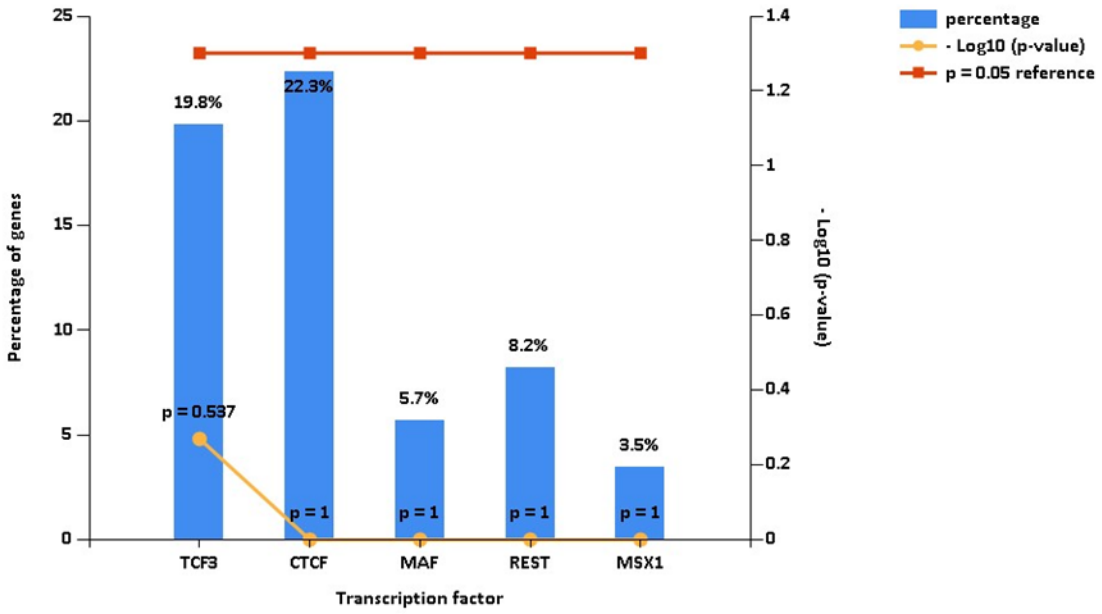

Figure 5.8. Funrich enriched transcription factors of genes from BeWo cells treated with 10 nM BPA.

5.2.1.2 Gene expression validation of microarray in non-syncytialised cells

In order to validate the outcome of the microarray, genes of interest were picked from the upregulated gene cohort, and upregulated gene expression was further assessed using qPCR. Genes that were chosen for validation were chosen due to their possible relation to placental function or cell function and were all significantly upregulated. Of the top upregulated genes in each cohort, 3 genes were chosen for validation. The genes chosen were: caveolin1 (CAV1), Leptin, and human placenta specific 8 (hPLAC8). These three genes were significantly upregulated in both 3 nM and 10 nM BPA treated non-syncytialised BeWo cells. Furthermore, immunofluorescent staining was performed on these three proteins in order to assess protein expression.

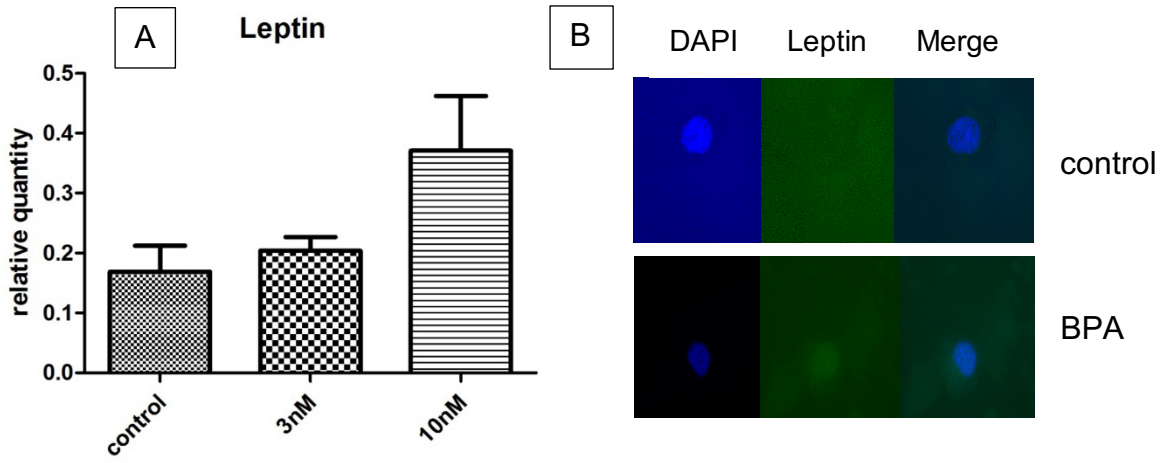

Figure 5.9. Panel a: there was a relative increase in leptin gene expression when compared to controls after 10 nM BPA treatment. This is in accordance with the fold-change difference (2.22) found between untreated BeWo cells and 10 nM BPA treated BeWo cells found in microarray analysis. Panel b: immunostaining shows an increase in Leptin levels after treatment with BPA.

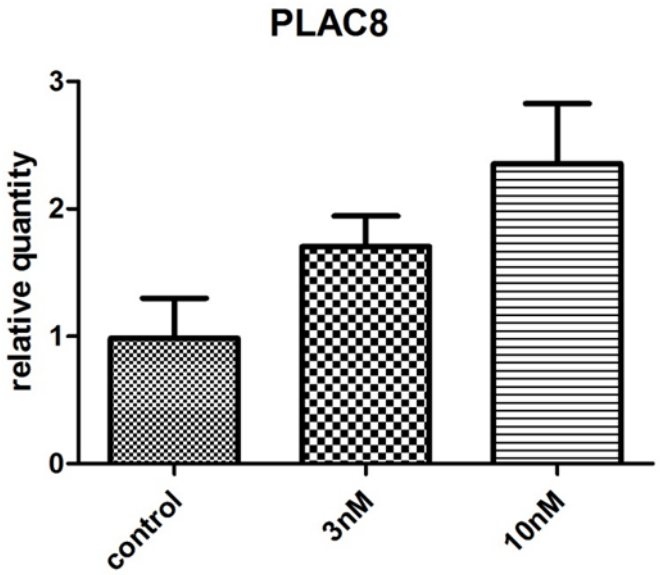

Figure 5.10. There was a relative increase in PLAC8 gene expression when compared to controls after 3 nM and 10 nM BPA treatment. This is in accordance with the fold-change difference found between untreated BeWo cells and 3 nM BPA treated BeWo cells (1.75) as well as untreated BeWo cells and 10 nM treated BeWo cells (2.14). No immunostaining available as no commercial antibodies available.

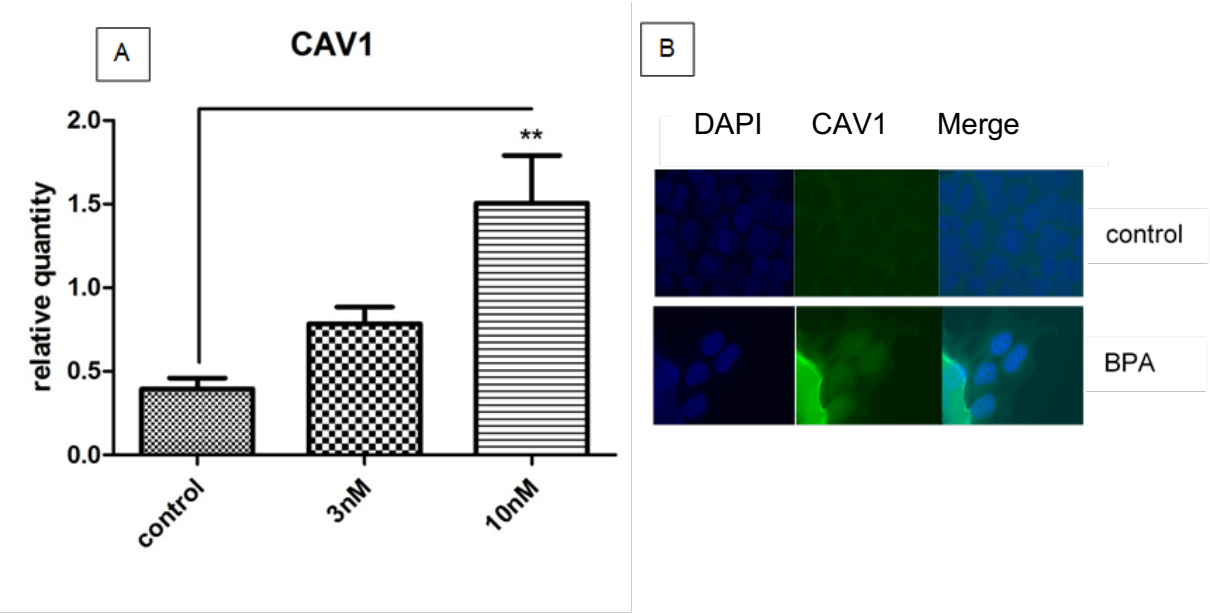

Figure 5.11. Panel a: there was a relative increase in CAV1 gene expression when compared to controls after 3 nM and 10 nM BPA treatment. There is a significant increase in CAV1 when comparing controls to 10 nM treated BeWo cells ($P<0.01$). This is in accordance with the fold-change difference found between untreated BeWo cells and 3 nM BPA treated BeWo cells (2.35) as well as untreated BeWo cells and 10 nM treated BeWo cells (3.55). Panel b: immunostaining shows an increase in CAV1 levels after treatment with BPA.

5.2.1.3 Bioinformatic analysis of syncytialised BeWo cells treated with BPA

Syncytialised BeWo cells were analysed separately in order to asses significantly differentially regulated genes and their functions as well as involvement in cell signaling pathways and diseases. Similarly, as in non-syncytialised BeWo cells, Venn diagrams were created to show overlap in differentially regulated genes. Figure 5.12 shows that in 3 nM syncytialised BeWo cells, 309 genes were differentially regulated, and in 10 nM syncytialised BeWo cells, 158 genes were differentially regulated, with only on gene, FABP5 being shared.

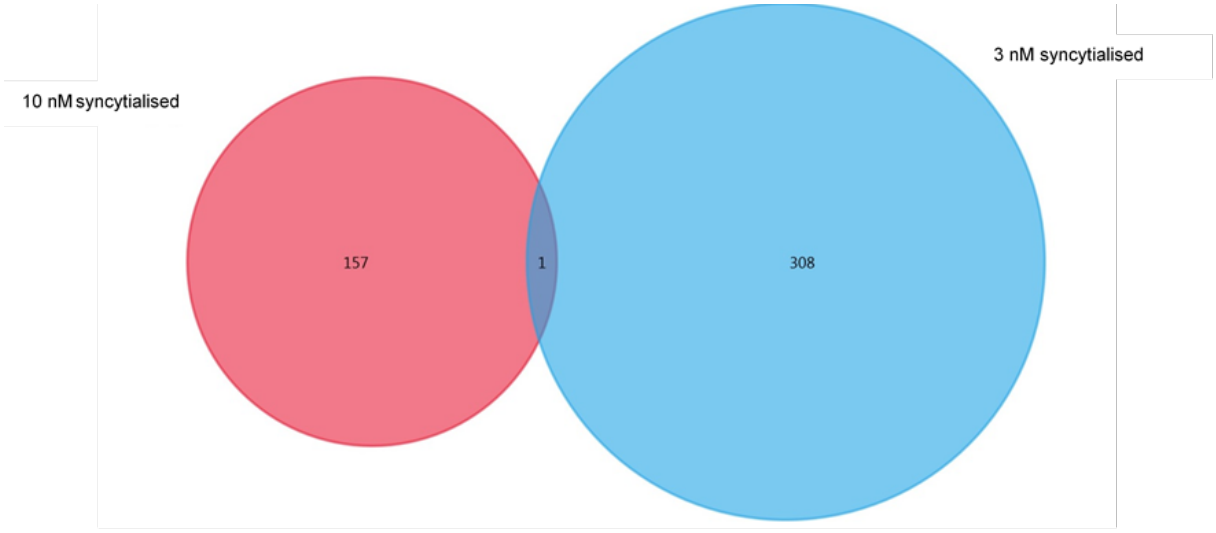

Figure 5.12. Venn diagram showing differentially expressed gene overlap between 10 nM syncytialised and syncytialised BeWo cells. In total, 1 gene was differentially expressed in both gene sets. The total set of differentially expressed genes was much larger in 3 nM non-syncytialised BeWo cells with a total number of 309 genes, compared to 158 genes. FABP5: shared differentially regulated gene.

Top upregulated genes in 3 nM treated syncytialised cells are shown below (table 5.13). They have been organised by highest to lowest signifciance (p-value) and their functions are described. The most significantly upregulated gene in this set is Growth hormone releasing hormone (GHRH). This protein stimulates growth hormone secretion.

GENE	FUNCTION	P-VALUE
Growth hormone releasing hormone (GHRH)	Stimulates growth hormone secretion	$5.69\ 10^{-4}$
UDP-glucuronosyltransferase 2B10 (UGT2B10)	Essential for conjugation and elimination of toxic xenobiotics and endogenous compounds	0.005
Carbonic anhydrase-related protein 11 (CA11)	unknown	0.005
Natural resistance-associated macrophage protein 1 (SLC11A1)	Metal transporter involved in iron metabolism. Controls infection due to macrophage-specific transport function.	0.01
Rab proteins geranylgeranyltransferase component A 1 (CHM)	Involved Rab protein geranylgeranylation	0.023
OTU domain-containing protein 7A (OTUD7A)	Deubiquinates polyubiquitin chains	0.023
Envoplakin-like protein (EVPLL)	Involved in intramedial filament binding and epidermis development	0.027
Schlafen-like protein 1 (SLFNL1)	unknown	0.038
Excitatory amino acid transporter 5 (SLC1A7)	Voltage-dependent transport of L-glutamate	0.038
Sulfotransferase 1C4 (SULT1C4)	catalyses sulfate conjugation of drugs, xenobiotic compounds, hormones, and neurotransmitters	0.042

Table 5.13. Top 10 upregulated genes in 3 nM BPA treated syncytialised BeWo cells including protein functions and p-values of upregulation. Where not cited, functions have been summarised from the UniProt protein database (https://www.uniprot.org). The most significantly upregulated gene is GHRH, followed by UGT2B10, CA11, SLC11A1, CHM, OTUD7A, EVPLL, SLFNL1, SLC1A7 and SULT1C4, respectively.

Top upregulated genes in 10 nM treated syncytialised cells are shown below (table 5.14). They have been organised by highest to lowest significance (p-value) and their functions are described. The most significantly upregulated gene in this set is Sodium-dependent phosphate transporter 2 (SLC20A2). This protein plays a role in phosphate housekeeping, signal transduction and cellular metabolism.

GENE	FUNCTION	P-VALUE
Sodium-dependent phosphate transporter 2 (SLC20A2)	Sodium-phosphate symporter. Plays role in phosphate housekeeping, signal transduction and cellular metabolism.	0.007
Probable tubulin polyglutamylase TTLL9 (TTLL9)	Likely to be a tubulin polyglutamylase forming polyglutamate side chains on tubulin.	0.007
Arginyl-tRNA--protein transferase 1 (ATE1)	Posttranslational conjugation of arginine in order to degrade via ubiquitine pathway.	0.008
Adhesion G protein-coupled receptor A2 (GPR124)	G-protein receptor of endothelium. Activates canonical Wnt signaling. Plays a major role in blood-brain barrier formation.	0.010
Golgi-associated plant pathogenesis-related protein 1 (GLIPR2)	Regulates type 1 Interferon signaling activity (Q. Zhou, Hao, Huang, & Cai, 2016).	0.012
Tumor necrosis factor receptor superfamily member 6 (FAS)	TNFSF6/FASLG complex receptor recruiting caspase-8. Resulting cell death-inducing complex results in series of caspase activations leading to apoptosis.	0.012
Multiple inositol polyphosphate phosphatase 1 (MINPP1)	Acts as a phosphoinositide 5- and phosphoinositide 6-phosphatase. Plays a role in bone development.	0.015
Protein transport protein Sec61 subunit gamma (SEC61G)	Plays a role in endoplasmic reticulum protein translocation	0.015
Sprouty-related, EVH1 domain-containing protein 1 (SPRED1)	Inhibits the activation of MAP kinase by growth factors. Inhibits hematopoiesis in bone marrow.	0.017
Carcinoembryonic antigen-related cell adhesion molecule 3 (CEACAM3)	Granulocyte receptor. Mediates phagocytosis of microorganisms, playing a role in innate immune system. Stimulates RAC1.	0.017

Table 5.14. Top 10 upregulated genes in 10 nM BPA treated syncytialised BeWo cells including protein functions and p-values of upregulation. Where not cited, functions have been summarised from the UniProt protein database (https://www.uniprot.org). The most significantly upregulated gene is SLC20A2, followed by TTLL9, ATE1, GPR124, GLIPR2, FAS, MINPP1, SEC61G, SPRED1 and CEACAM3, respectively.

Next, gene enrichment analysis was performed in non-syncytialised BeWo cells treated with 3 nM and 10 nM BPA. In cells treated with 3 nM BPA, the Funrich molecular function database did not show any significant gene enrichment (figure 5.13), however using the Go Molecular Function 2017b database via Enrichr did yield

significant results (table 5.15). Funrich analysis for biological processes (figure 5.14) did not yield any significant results, but Enrichr data using the Go Biological Process 2017b database (table 5.16) did show significant gene enrichment. Biological pathway enrichment analysis also did not show significance when using the Funrich database (figure 5.15), however the Biocarta 2016 database via Enrichr showed significant gene enrichment pathways, including apoptotic pathways (table 5.17). Funrich analysis for transcription factors (figure 5.16) also did not show any significant gene enrichment.

In syncytialised cells treated with 10 nM BPA, molecular functions analysed via Funrich did not show any significant gene enrichment (figure 5.17), but molecular functions analysed with the GO molecular function 2017b database via Enrichr showed several significantly enriched molecular functions (table 5.18). When analysing biological processes in 10 nM treated syncytialised cells, the Funrich database did not yield significant results (figure 5.18), however the analysis with Enrichr showed significant results via the GO biological process 2017b database (table 5.19), with a focus on DNA repair processes being three of the top 4 differentially regulated biological processes. When analysing biological pathways via Funrich (figure 5.19), no significant gene enrichment was found, however biological pathway analysis using the NCI Nature 2016 database via Enrichr (table showed significantly regulated gene enrichment in biological processes, with a focus on pathways stimulating apoptosis such as direct p53 effectors and FAS signaling pathway. There were no significantly enriched transcription factors via the Funrich database (figure 5.20).

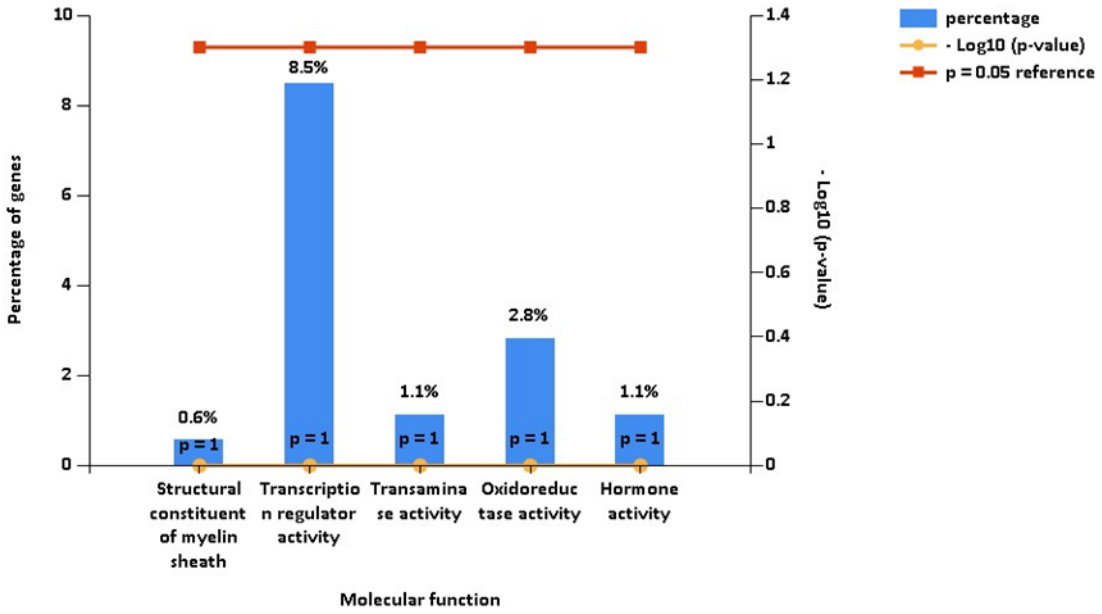

Figure 5.13. Funrich enriched molecular functions of genes from syncytialised BeWo cells treated with 3 nM BPA.

Index	Name	P-value
1	DNA replication origin binding (GO:0003688)	0.01659
2	interleukin-21 receptor activity (GO:0001532)	0.02188
3	CTD phosphatase activity (GO:0008420)	0.04415
4	amino acid-transporting ATPase activity (GO:0015424)	0.04415
5	hydroxyjasmonate sulfotransferase activity (GO:0080131)	0.04486
6	thiol sulfotransferase activity (GO:0033870)	0.04486
7	chondroitin sulfotransferase activity (GO:0034481)	0.04486
8	flavonoid sulfotransferase activity (GO:1990135)	0.04486
9	11-hydroxyjasmonate sulfotransferase activity (GO:0102056)	0.04486
10	brassinosteroid sulfotransferase activity (GO:0080118)	0.04486

Table 5.15. Top 10 significant molecular functions associated with differentially expressed genes after 3 nM BPA treatment of syncytialised BeWo cells using Go Molecular Function 2017b database via Enrichr.

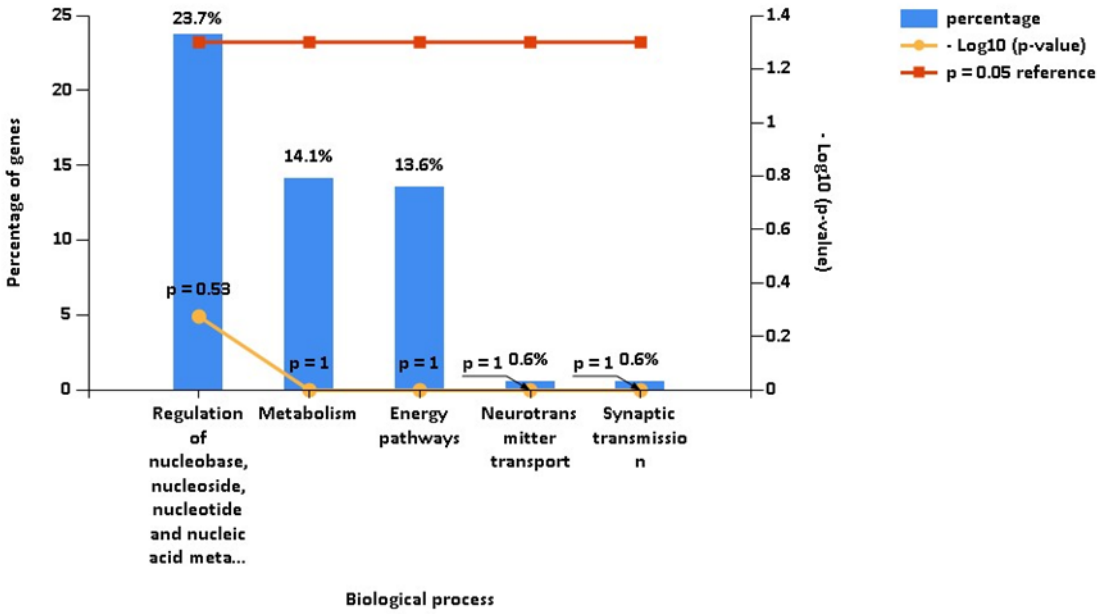

Figure 5.14. Funrich enriched biological processes of genes from syncytialised BeWo cells treated with 3 nM BPA.

Index	Name	P-value
1	natural killer cell differentiation (GO:0001779)	0.003426
2	positive regulation of protein serine/threonine phosphatase activity (GO:1905184)	0.007973
3	positive regulation of protein tyrosine phosphatase activity (GO:1903615)	0.007973
4	modulation by virus of host protein transport (GO:0044833)	0.009865
5	positive regulation of thymocyte migration (GO:2000412)	0.01194
6	positive regulation of myosin-light-chain-phosphatase activity (GO:0035508)	0.01194
7	intestinal epithelial structure maintenance (GO:0060729)	0.01418
8	maintenance of gastrointestinal epithelium (GO:0030277)	0.01418
9	mannose to fructose-6-phosphate metabolic process (GO:0061611)	0.01659
10	telomere maintenance via telomere lengthening (GO:0010833)	0.01660

Table 5.16. Top 10 significant molecular functions associated with differentially expressed genes after 3 nM BPA treatment of syncytialised BeWo cells using Go Biological Process 2017b database via Enrichr.

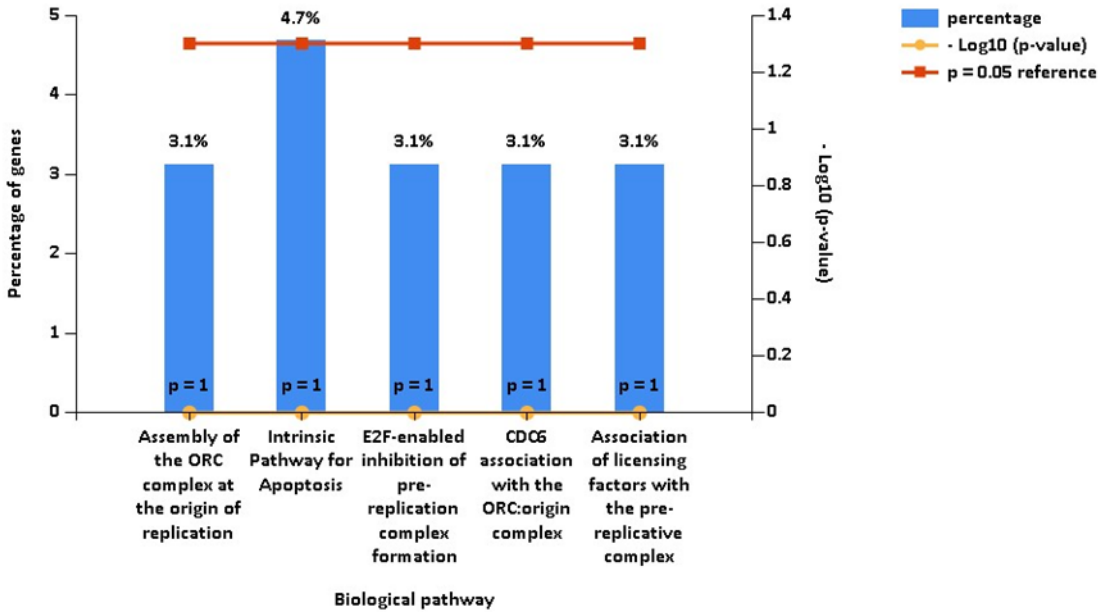

Figure 5.15. Funrich enriched biological pathways of genes from syncytialised BeWo cells treated with 3 nM BPA.

Index	Name	P-value
1	Role of Mitochondria in Apoptotic Signaling_Homo sapiens_h_mitochondriaPathway	0.01659
2	Apoptotic Signaling in Response to DNA Damage_Homo sapiens_h_chemicalPathway	0.01915
3	Stress Induction of HSP Regulation_Homo sapiens_h_hsp27Pathway	0.01915
4	CDK Regulation of DNA Replication_Homo sapiens_h_mcmPathway	0.03093
5	Multi-Drug Resistance Factors_Homo sapiens_h_mrpPathway	0.08920
6	Ceramide Signaling Pathway_Homo sapiens_h_ceramidePathway	0.09189
7	Overview of telomerase RNA component gene hTerc Transcriptional Regulation_Homo sapiens_h_tercPathway	0.1033
8	Pertussis toxin-insensitive CCR5 Signaling in Macrophage_Homo sapiens_h_Ccr5Pathway	0.1308
9	Role of PPAR-gamma Coactivators in Obesity and Thermogenesis_Homo sapiens_h_ppargPathway	0.1308
10	West Nile Virus_Homo sapiens_h_WNVpathway	0.1308

Table 5.17. Top 10 significant biological pathways associated with differentially expressed genes after 3 nM BPA treatment of syncytialised BeWo cells using Biocarta 2016 database via Enrichr. The top two significant pathways involve apoptosis signaling (p=0.017, p=0.019 respectively).

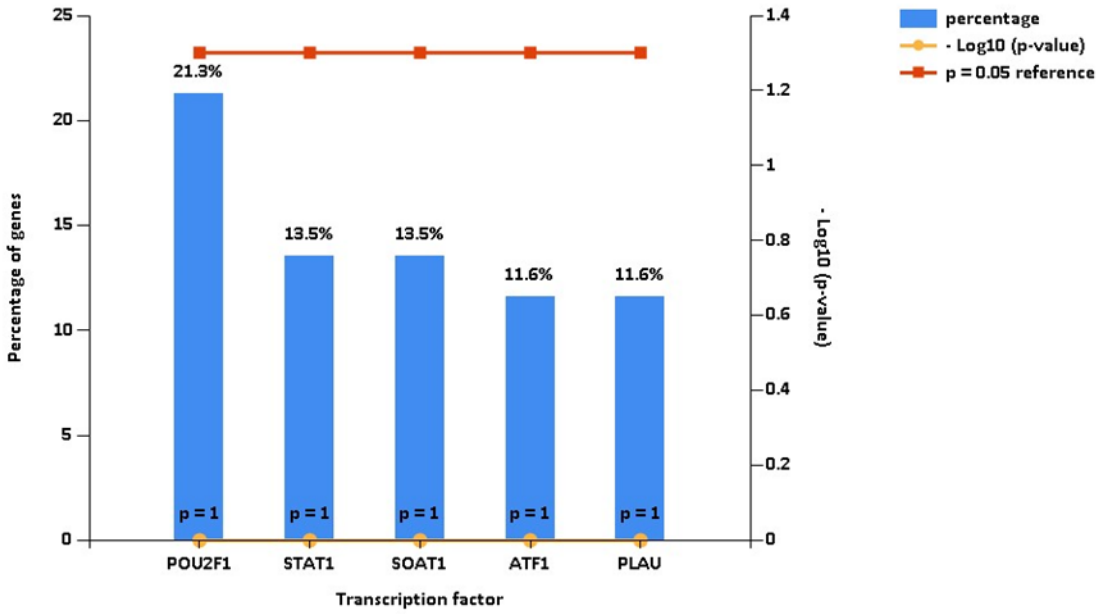

Figure 5.16. Funrich enriched transcription factors of genes from syncytialised BeWo cells treated with 3 nM BPA.

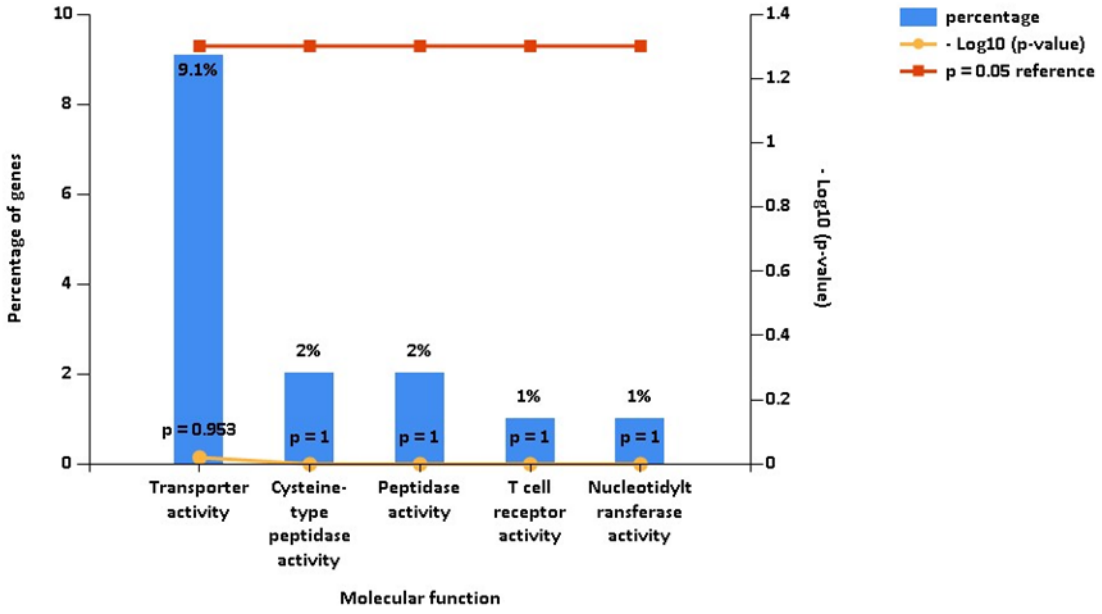

Figure 5.17. Funrich enriched molecular functions of genes from syncytialised BeWo cells treated with 10 nM BPA.

Index	Name	P-value
1	ankyrin binding (GO:0030506)	0.0003618
2	lipoate-protein ligase activity (GO:0016979)	0.001683
3	protein kinase binding (GO:0019901)	0.02202
4	phosphatase binding (GO:0019902)	0.03977
5	beta-glucuronidase activity (GO:0004566)	0.04648
6	membrane insertase activity (GO:0032977)	0.04921
7	protein transporter activity (GO:0008565)	0.04921
8	cytidylyltransferase activity (GO:0070567)	0.05401
9	aldose-1-phosphate nucleotidyltransferase activity (GO:0047347)	0.05401
10	adenylyltransferase activity (GO:0070566)	0.05401

Table 5.18. Top 10 significant molecular functions with differentially expressed genes after 10 nM BPA treatment of syncytialised BeWo cells using GO Molecular Function 2017b database via Enrichr.

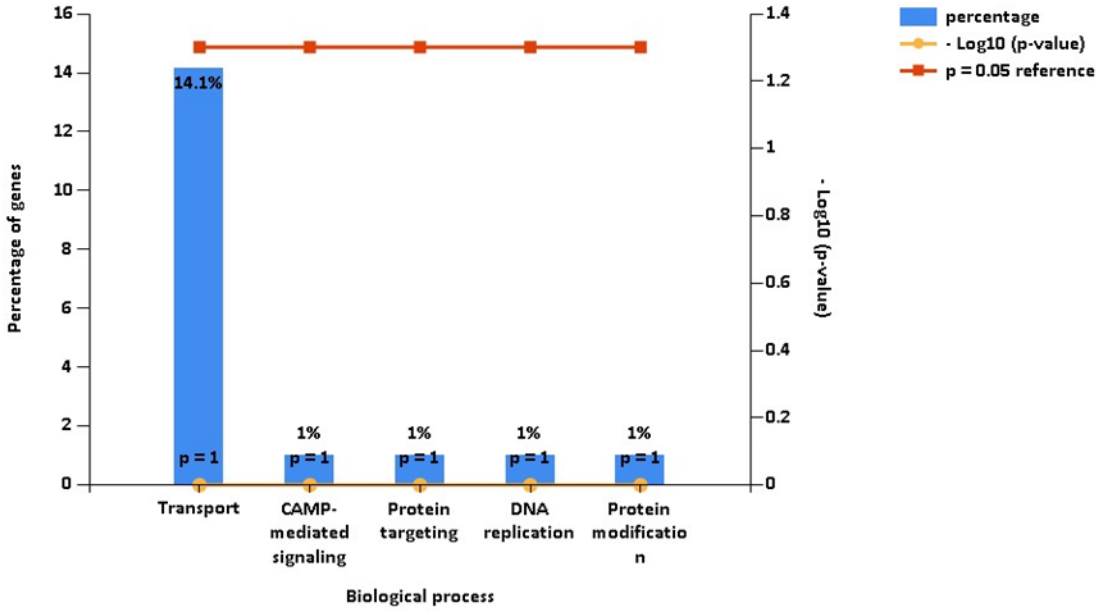

Figure 5.18. Funrich enriched biological processes of genes from syncytialised BeWo cells treated with 10 nM BPA.

Index	Name	P-value
1	DNA synthesis involved in double-strand break repair via single-strand annealing (GO:0043151)	0.002419
2	translesion synthesis (GO:0019985)	0.002949
3	DNA synthesis involved in double-strand break repair via homologous recombination (GO:0043150)	0.004820
4	DNA synthesis involved in UV-damage excision repair (GO:1904161)	0.005054
5	dentin secretion (GO:0070468)	0.006920
6	tooth placode formation (GO:0060790)	0.006920
7	protein targeting to lysosome involved in chaperone-mediated autophagy (GO:0061740)	0.008733
8	positive regulation of peptidyl-serine phosphorylation (GO:0033138)	0.008895
9	positive regulation of interferon-gamma-mediated signaling pathway (GO:0060335)	0.01180
10	induction by virus of host protein phosphorylation (GO:0039614)	0.01241

Table 5.19. Top 10 significant biological processes with differentially expressed genes after 10 nM BPA treatment of syncytialised BeWo cells using GO Biological Process 2017b database via Enrichr. Three of the most significant biological processes have to do with DNA repair: index 1 (p=0.002), index 2 (p=0.005), and index 4 (p=0.005).

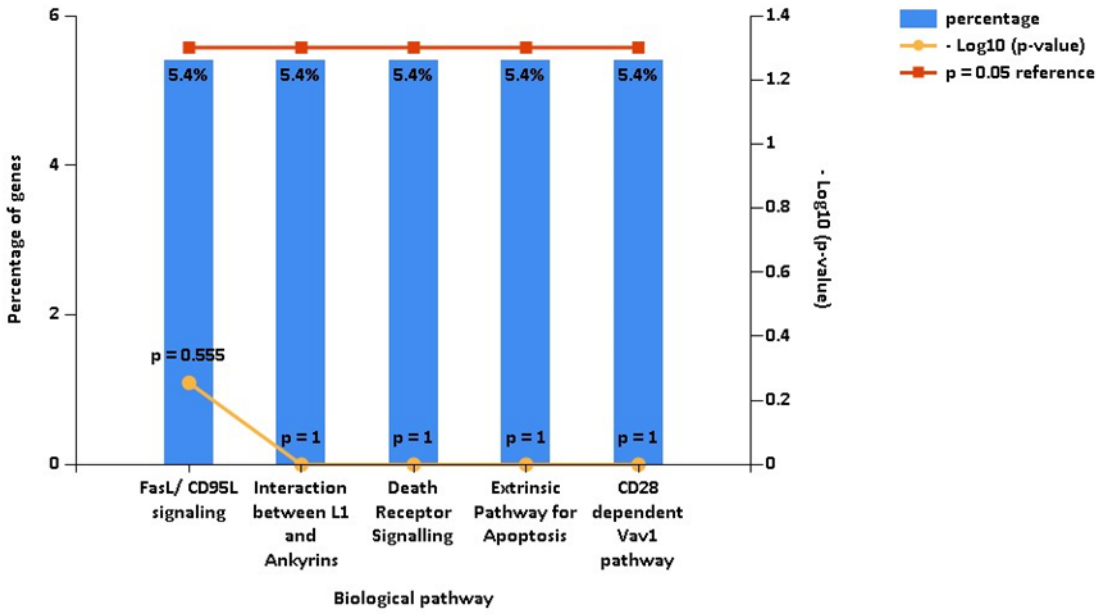

Figure 5.19. Funrich enriched biological pathways of genes from syncytialised BeWo cells treated with 10 nM BPA.

Index	Name	P-value
1	Direct p53 effectors_Homo sapiens_67c3b75d-6191-11e5-8ac5-06603eb7f303	0.02290
2	FAS (CD95) signaling pathway_Homo sapiens_79cc9c14-6192-11e5-8ac5-06603eb7f303	0.03621
3	Caspase Cascade in Apoptosis_Homo sapiens_b9d3ef2e-618f-11e5-8ac5-06603eb7f303	0.07018
4	AP-1 transcription factor network_Homo sapiens_3ce2f9c5-6189-11e5-8ac5-06603eb7f303	0.1033
5	Degradation of beta catenin_Homo sapiens_61b6f2fc-6191-11e5-8ac5-06603eb7f303	0.1262
6	Hypoxic and oxygen homeostasis regulation of HIF-1-alpha_Homo sapiens_4c0f3584-6193-11e5-8ac5-06603eb7f303	0.1331
7	Canonical Wnt signaling pathway_Homo sapiens_9dbe253d-618f-11e5-8ac5-06603eb7f303	0.1468
8	ALK1 signaling events_Homo sapiens_fcc72679-6186-11e5-8ac5-06603eb7f303	0.1800
9	VEGFR1 specific signals_Homo sapiens_8b13143b-6196-11e5-8ac5-06603eb7f303	0.1929
10	TRAIL signaling pathway_Homo sapiens_3a79fddf-6196-11e5-8ac5-06603eb7f303	0.1993

Table 5.20. Top 10 significant biological pathways with differentially expressed genes after 10 nM BPA treatment of syncytialised BeWo cells using NCI Nature 2016 database via Enrichr. Apoptosis related signaling pathways are the top two significantly enriched pathways: index 1, direct p53 effectors (p=0.023); index 2, FAS signaling pathway (p=0.036).

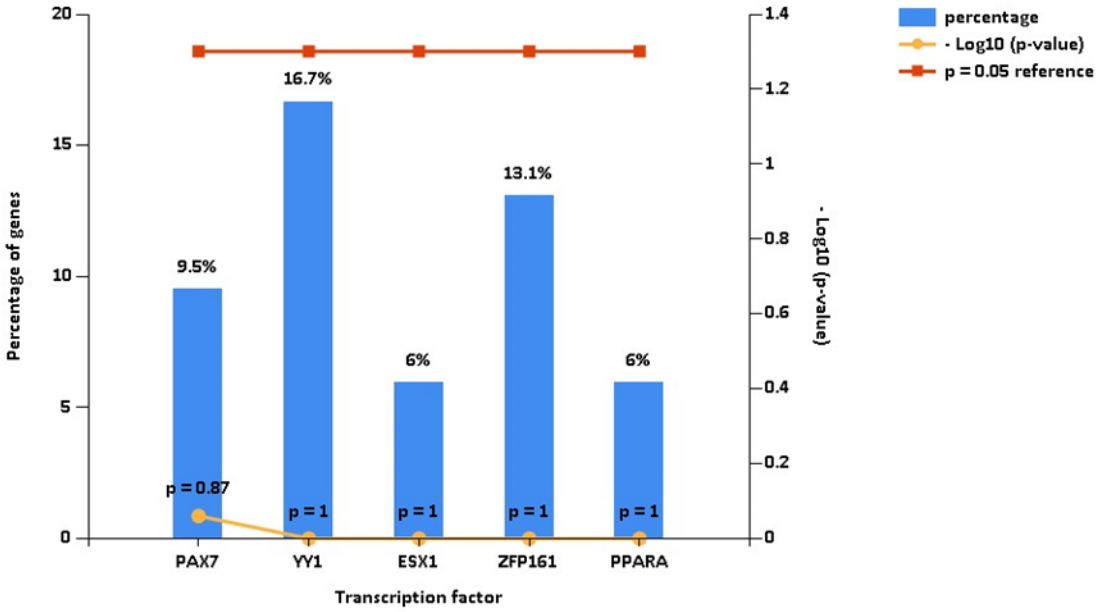

Figure 5.20. Funrich enriched transcription factors of genes from syncytialised BeWo cells treated with 10 nM BPA.

5.2.1.4 Gene expression validation of microarray in syncytialised cells

Immunostaining was performed on genes that were upregulated in both 3 nM BPA treated and 10 nM BPA treated BeWo cells in order to validate expression on the protein level. 2 genes, cytoplasmatic-linker associated protein 2 (CLASP2) and single-minded homolog 2 (SIM2) were chosen due to their relevance to placental physiology and/or developmental relevance. Figure 5.21 shows images of immunofluorescent staining of 10 nM treated syncytialised BeWo cells with CLASP2. After 24 hours, there does not appear to be an upregulation of CLASP2 on the protein level. Figure 5.22 shows images of immunofluorescent staining of 10 nM treated syncytialised BeWo cells with SIM2. After 24 hours, there appears to be an upregulation of SIM2 on the protein level.

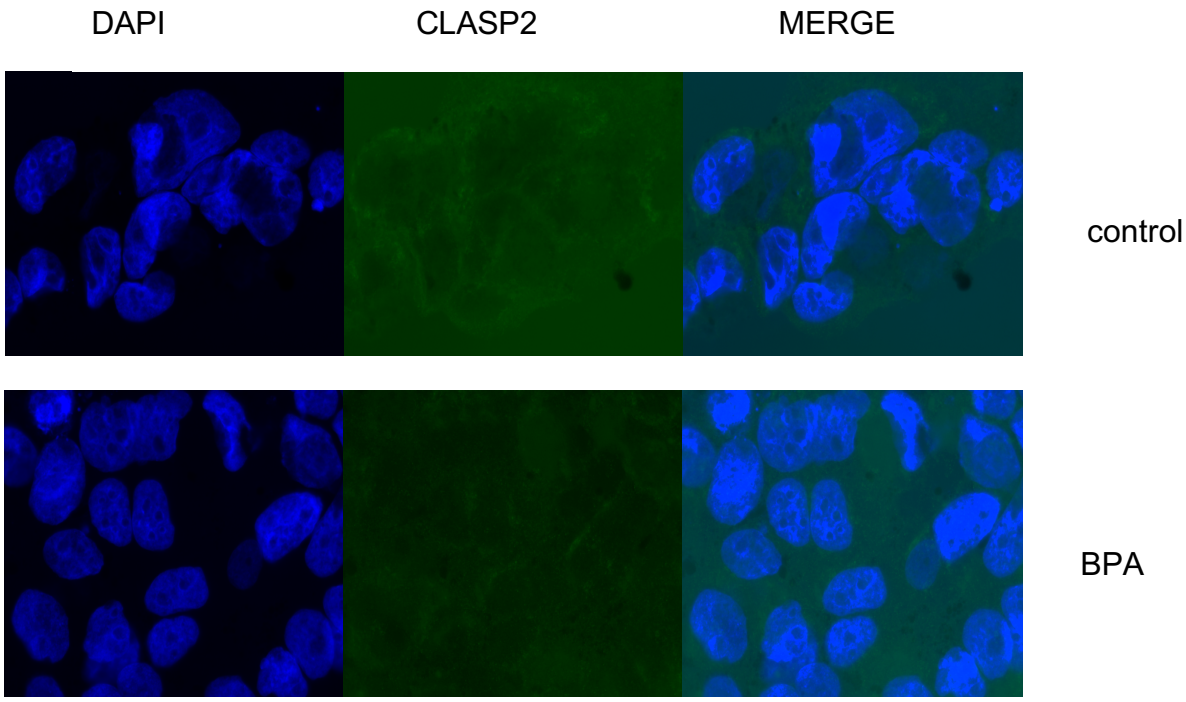

Figure 5.21. Immunostaining of CLASP2 protein in syncytialised BeWo cells treated with 10 nM BPA. There does not appear to be an upregulation of CLASP-2 on protein levels after 24 hour treatment with BPA.

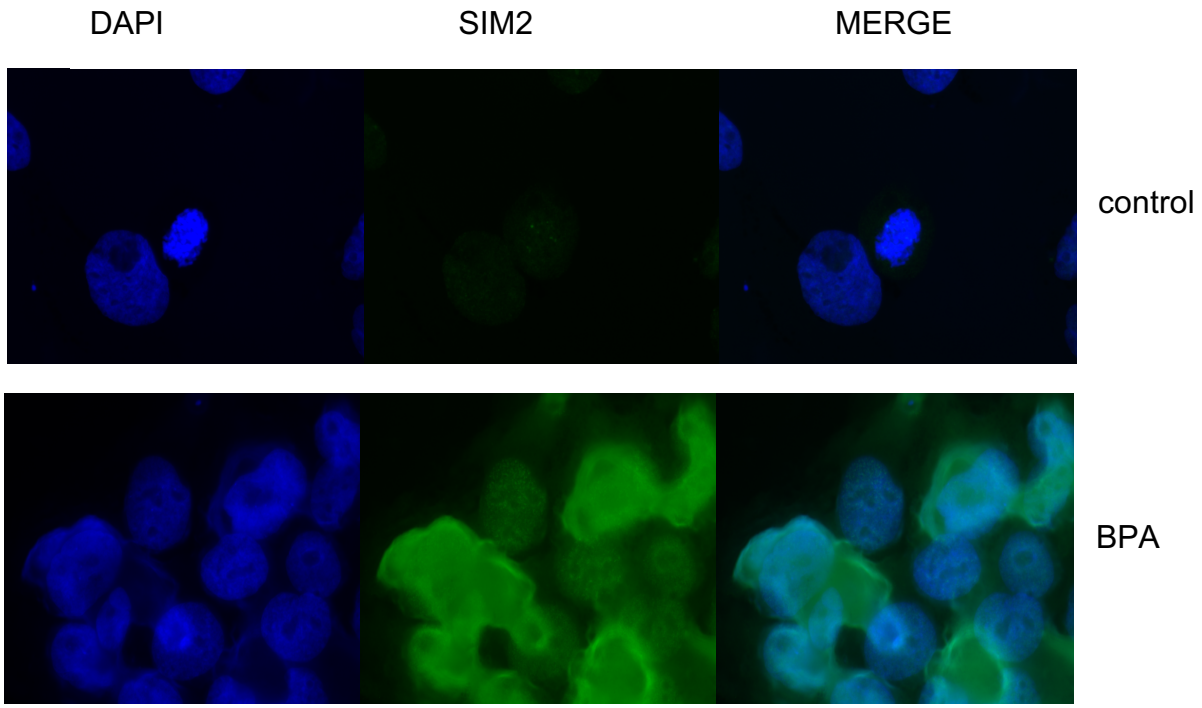

Figure 5.22. Immunostaining of SIM2 protein in syncytialised BeWo cells treated with 10 nM BPA. There appears to be an upregulation of SIM2 on protein levels after 24 hour treatment with BPA.

Pathways and diseases that were significantly upregulated were found throughout all treatments, but were most significantly found in 3 nM non-syncytialised BPA treated BeWo cells. Guanyl-nucleotide exchange factor activity was the most significantly altered molecular function according to Funrich (adjusted p-value= 0.049). When further analysing molecular function using the Enrichr tool, Rac-guanyl nucleotide exchange factor and Rho-guanyl nucleotide exchange factor activity were the top enriched Go-terms associated with 3 nM BPA treatment in non-syncytialised BeWo cells (p= 0.00009435 and 0.0002070 respectively, GO molecular function 2017b). Similarly, regulation of Rac1 and regulation of RhoA, as well as Rac1 signaling and RhoA signalling were significantly enriched within the category of biological pathways (adjusted p-value= 0.007). Using the Enrichr tool for further bioinformatics analysis yielded gene enrichment within the insulin resistance pathway (p= 0.01312, NCI Nature 2016 database), as well as regulation of RhoA activity (p= 0.0003159, KEGG 2015 database) and RhoA signalling pathway (p= 0.05999, KEGG 2015 database) and differentiation of white and brown adipocyte (p= 0.001369, WikiPathways 2016), insulin signalling (p= 0.03006, WikiPathways 2016) and leptin signalling pathway (p= 0.03426, WikiPathways 2016). Within the 3 nM non-syncytialised BeWo group,

RREB1 was the most enriched transcription factor using the Funrich tool (adjusted p-value 0.004). This transcription factor was also found to be enriched using the Enrichr tool for (p= 0.007083, ARCHS4 TFs Coexp database). Within the group of 10 nM BPA treated non-syncytialised BeWo cells Funrich showed no significant enrichment, however Enrichr showed that insulin resistance biological pathways were enriched (p=0.0054, KEGG 2016 database) as well as differentiation of white and brown adipocyte (p= 0.002715, WikiPathways 2016 database) and regulation of RAC1 activity (p=0.01243, NCI-Nature 2016 database), similar to 3 nM BPA treated non-syncytialised cells. Within syncytialised BeWo cells, both 3 nM treatments and 10 nM treatments showed no significant enriched terms for any function in Funrich, however 10 nM syncytialised cells showed a strong trend towards FasL/CD95L signalling pathway function in Funrich, which was confirmed using Enrichr. Using Enrichr, direct p53 effectors as well as the FasL/CDC9L signalling pathway were significantly enriched according to the NCI Nature 2016 database. These findings indicate that higher levels of BPA may have a toxic and pro-apoptotic effect on syncytialised, but not on non-syncytialised BeWo cells.

5.2.2. Bioinformatic analysis of placental microarray results

To test whether BPA treatment resulted in similar gene expression changes in human placental cells, human placental explants were treated with 3 nM and 10 nM BPA for 24 hours, after which RNA was extracted, and cDNA prepared to analyse using microarray as previously described. Placenta was retrieved from a natural term birth with no gestational complications.

As with BeWo cells, it was important to use a non-biased screen such as microarray analysis, in order to evaluate gene expression changes after BPA treatment in the entire human placental genome. It was important to assess which significant changes were present after BPA treatment, and whether these changes echoed the findings in BeWo cells treated with BPA, as these cells are a model of the human placenta.

When analysing biological pathways in 10 nM BPA treated placental explants compared to controls (Figure 5.23), Funrich data showed a significant enrichment in AlphaE beta7 integrin cell surface interactions (p<0.001). When using the CORUM

database via Enrichr (table 5.21), pathways such as caveolar macromolecular signaling complex (p=0.005) were differentially regulated, which is significant as CAV1 was a significantly upregulated protein in BPA treated BeWo cells. Clinical phenotypes analysed using the Funrich database (figure 5.24) did not show any significance, however analysis using the MGI mammalian phenotype via Enrichr (table 5.22) showed significantly regulated enriched phenotypes including increased percent body fat/body weight (p=0.39) and increased circulating LDL cholesterol level (p=0.029), pathways and7or phenotypes which were found in BeWo cells treated with BPA. When using the dbGAP database via Enrichr for the analysis of phenotype (table 5.23), one of the significantly enriched phenotypes was diabetes mellitus (p=0.042), a differentially regulated disease in BPA treated BeWo cells.

When comparing 10 nM to 3 nM treatment in placental explants, Funrich data did not show any level of significance (figure 5.25 and 5.26). When using the KEGG 2016 database via Enrichr (table 5.24), there was a significant regulation in the insulin secretion pathway (p=0.009), which was differentially regulated in BPA treated BeWo cells as well. Tables 5.25, 5.26 and 5.27 show significantly regulated mammalian phenotypes using the MGI mammalian phenotype database via Enrichr. Table 5.25 shows a significant regulation of decreased insulin secretion (p=0.009), table 5.26 shows significant regulation of (abnormal) gluconeogenesis (p=0.017) and hyperglycemia (p=0.025) and table 5.27 shows significant regulation of (impaired) glucose tolerance (p=0.031). These phenotypes are related to diabetes, which was a differentially regulated pathway and/or phenotype in BPA treated BeWo cells. Lastly, table 5.28 shows differentially regulated diseases when comparing 10 nM and 3 nM BPA treated placental explants and shows that insulin resistance was differentially regulated (p=0.015), also being linked to diabetes.

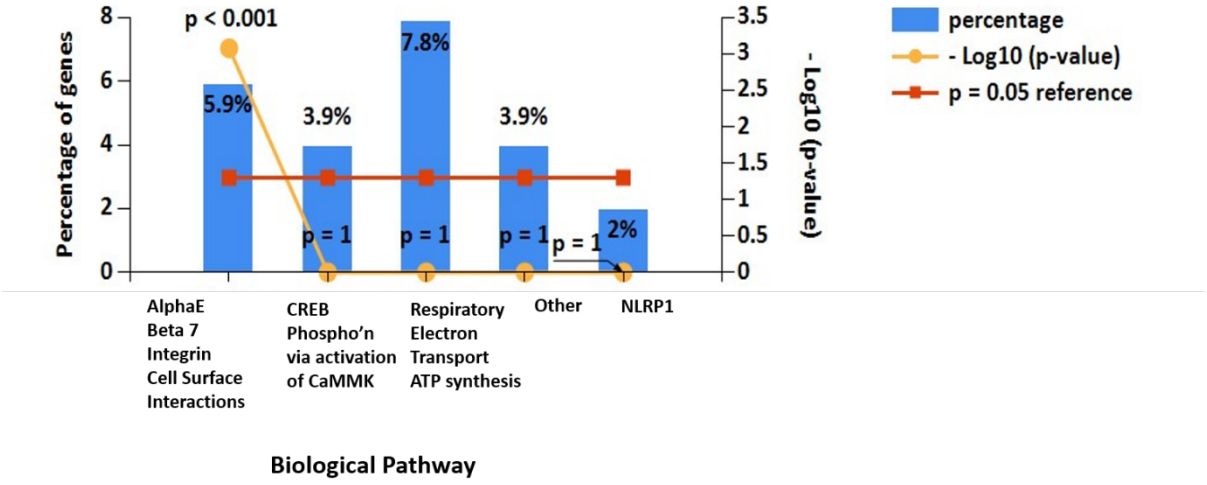

Figure 5.23 Funrich enriched biological pathways of genes from human placental explants treated with 10 nM BPA. AlphaE beta7 integrin cell surface interactions are significantly regulated ($p<0.001$).

Index	Name	P-value
1	CDH1-CKS1B complex (human)	0.003167
2	SEMA4A-PlexinD1 complex (human)	0.003167
3	SNX complex (SNX1, SNX6) (human)	0.003167
4	Caveolar macromolecular signaling complex (mouse)	0.005249
5	G protein complex (Hdac5, Gnb1, Gng2) (mouse)	0.009145
6	NCOR-SIN3-HDAC1 complex (human)	0.009145
7	PSD95-FYN-NR2A complex (human)	0.009145
8	Smrt-Sin3A-Hdac7 complex (mouse)	0.009145
9	HERP1/HEY2-NCOR-SIN3A complex (human)	0.01761
10	NCOR-SIN3-RPD3 complex (human)	0.01761

Table 5.21. Top 10 significant pathways with differentially expressed genes after 10 nM BPA treatment of placental explants using CORUM database via Enrichr. One of the top upregulated pathways (index number 4) was caveolar macromolecular signaling. Caveolar macromolecular signaling complex is differentially regulated (Index 4), and caveolin-1 was upregulated in BPA treated BeWo cells.

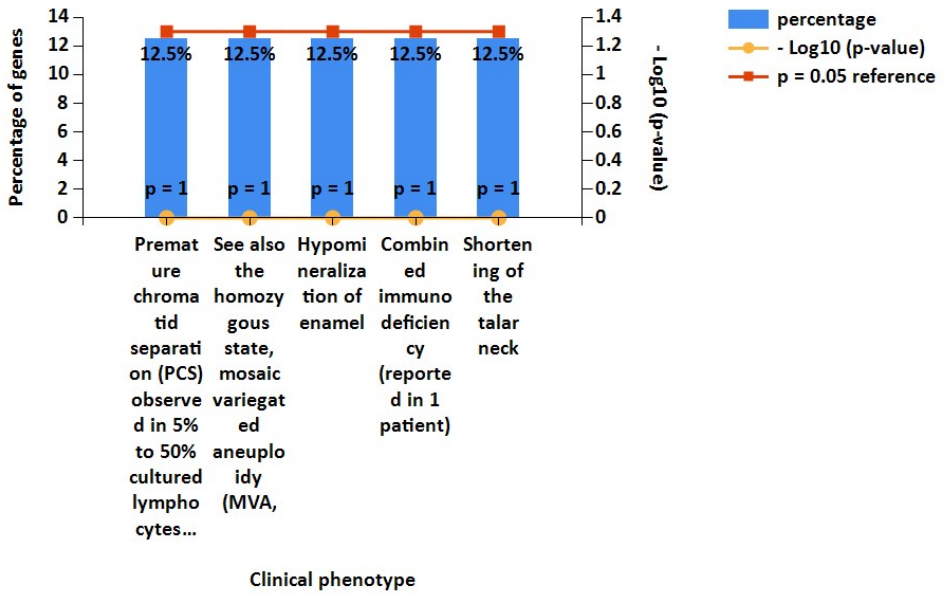

Figure 5.24 Funrich enriched clinical phenotypes of genes from human placental explants treated with 10 nM BPA.

Index	Name	P-value
11	MP:0009363_abnormal_secondary_ovarian_follicle_morphology	0.02671
12	MP:0005458_increased_percent_body_fat/body_weight	0.03922
13	MP:0005450_abnormal_energy_expenditure	0.03246
14	MP:0000182_increased_circulating_LDL_cholesterol_level	0.02908
15	MP:0003227_abnormal_vascular_branching_morphogenesis	0.03246
16	MP:0003886_abnormal_embryonic_epiblast_morphology	0.04014
17	MP:0001433_polyphagia	0.04709
18	MP:0001745_increased_circulating_corticosterone_level	0.04965
19	MP:0009339_decreased_splenocyte_number	0.04317
20	MP:0000703_abnormal_thymus_morphology	0.06060

Table 5.22. Top 11-20 mammalian phenotypes with differentially expressed genes after 10 nM BPA treatment of placental explants using MGI Mammalian Phenotype database via Enrichr. Index number 12: increased percent body fat/body weight. Index number 14: increased circulating LDL cholesterol level. Similar phenotypes and/or pathways were found in BeWo cells treated with BPA.

Index	Name	P-value
1	Leukemia, Lymphoid	0.02671
2	Diabetes Mellitus	0.04242
3	Attention Deficit Disorder with Hyperactivity	0.05190
4	Lipids	0.05577
5	Triglycerides	0.09568
6	Death, Sudden, Cardiac	0.05563
7	Inflammatory Bowel Diseases	0.1320
8	Amyotrophic Lateral Sclerosis	0.1353
9	Metabolic Syndrome X	0.1368
10	Bipolar Disorder	0.2161

Table 5.23. Top 10 diseases with differentially expressed genes after 10 nM BPA treatment of placental explants using dbGAP database via Enrichr. Index number 2: Diabetes Mellitus. Diabetes was a differentially regulated disease in BPA treated BeWo cells.

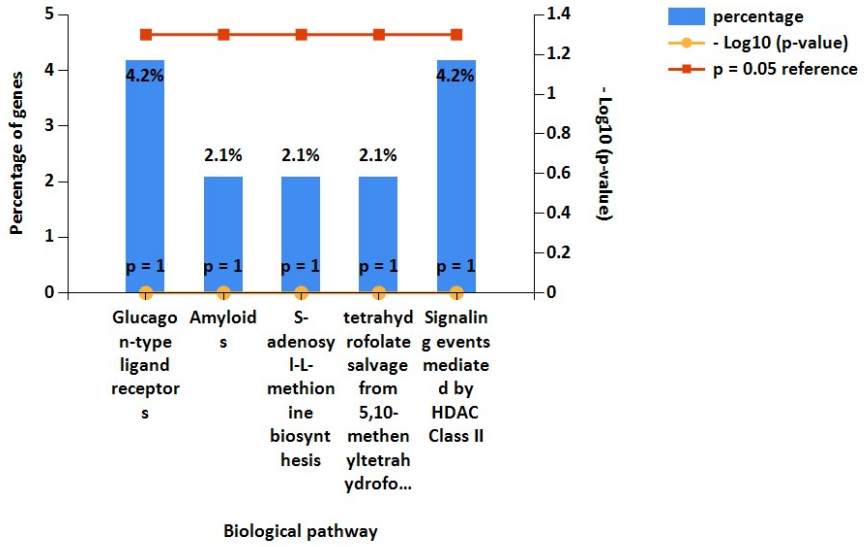

Figure 5.25 Funrich enriched biological pathways of genes from human placental explants comparing 10 nM BPA treatment to 3 nM BPA treatment.

Index	Name	P-value
1	Notch signaling pathway_Homo sapiens_hsa04330	0.006109
2	African trypanosomiasis_Homo sapiens_hsa05143	0.006835
3	Insulin secretion_Homo sapiens_hsa04911	0.009374
4	Alzheimer's disease_Homo sapiens_hsa05010	0.03298
5	Huntington's disease_Homo sapiens_hsa05016	0.03760
6	Renin secretion_Homo sapiens_hsa04924	0.07133
7	Non-homologous end-joining_Homo sapiens_hsa03450	0.07302
8	Calcium signaling pathway_Homo sapiens_hsa04020	0.09786
9	Systemic lupus erythematosus_Homo sapiens_hsa05322	0.09872
10	Base excision repair_Homo sapiens_hsa03410	0.1058

Table 5.24. Top 10 pathways with differentially expressed genes comparing 10 nM BPA treatment to 3 nM treatment in placental explants using KEGG 2016 database via Enrichr. Index number 3: Insulin secretion. This pathway was differentially regulated in BPA treated BeWo cells.

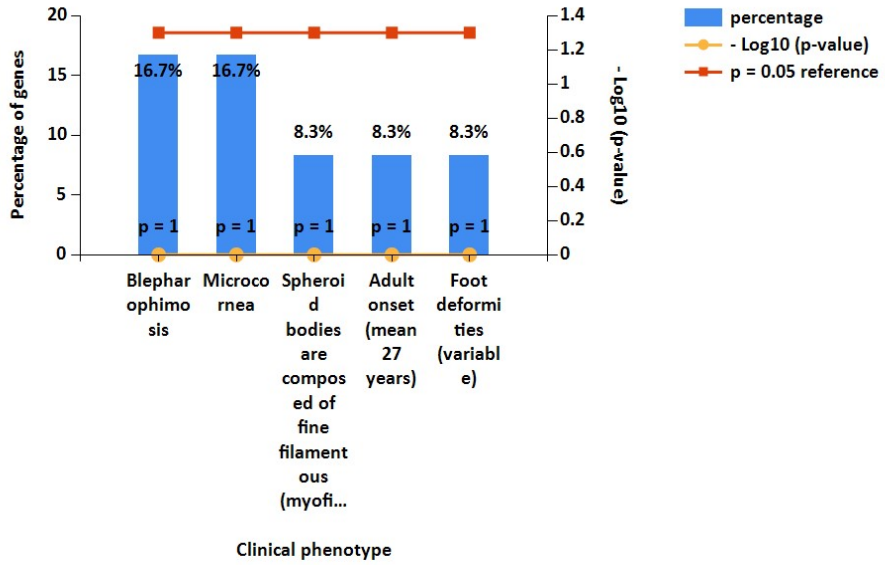

Figure 5.26 Funrich enriched clinical phenotypes of genes from human placental explants comparing 10 nM BPA treatment to 3 nM BPA treatment.

Index	Name	P-value
1	MP:0006029_abnormal_sclerotome_morphology	0.001095
2	MP:0004206_abnormal_dermomyotome_development	0.002053
3	MP:0009387_abnormal_epidermal_pigmentation	0.003534
4	MP:0002746_abnormal_semilunar_valve_morphology	0.003694
5	MP:0003886_abnormal_embryonic_epiblast_morphology	0.006027
6	MP:0010502_ventricle_myocardium_hypoplasia	0.006905
7	MP:0013600_testis_degeneration	0.007268
8	MP:0000281_abnormal_interventricular_septum_morphology	0.007463
9	MP:0003059_decreased_insulin_secretion	0.008750
10	MP:0000872_abnormal_cerebellum_external_granule_cell_layer_morphology	0.009706

Table 5.25. Top 10 mammalian phenotypes with differentially expressed genes comparing 10 nM BPA treatment to 3 nM treatment in placental explants using MGI Mammalian Phenotype database via Enrichr. Index number 9: decreased insulin secretion. Insulin signaling was differentially regulated in BPA treated BeWo cells.

Index	Name	P-value
21	MP:0003271_abnormal_duodenum_morphology	0.01669
22	MP:0003383_abnormal_gluconeogenesis	0.01773
23	MP:0010807_abnormal_stomach_position_or_orientation	0.02255
24	MP:0002659_pituitary_gland_hypoplasia	0.02255
25	MP:0001559_hyperglycemia	0.02530
26	MP:0003562_abnormal_pancreatic_beta_cell_physiology	0.02661
27	MP:0003721_increased_tumor_growth/size	0.02936
28	MP:0008501_increased_IgG2b_level	0.02936
29	MP:0012156_rostral-caudal_axis_duplication	0.02938
30	MP:0005085_abnormal_gallbladder_physiology	0.02938

Table 5.26. Top 21-30 mammalian phenotypes with differentially expressed genes comparing 10 nM BPA treatment to 3 nM treatment in placental explants using MGI Mammalian Phenotype database via Enrichr. Index number 22: abnormal gluconeogenesis. Index number 25: hyperglycemia. Glucose metabolism was differentially regulated in BPA treated BeWo cells.

Index	Name	P-value
31	MP:0002560_arrhythmic_circadian_persistence	0.02938
32	MP:0000238_absent_pre-B_cells	0.02938
33	MP:0005293_impaired_glucose_tolerance	0.03058
34	MP:0003448_abnormal_tumor_morphology	0.03066
35	MP:0000288_abnormal_pericardium_morphology	0.03066
36	MP:0003403_absent_placental_labyrinth	0.03066
37	MP:0000757_herniated_abdominal_wall	0.03066
38	MP:0006092_abnormal_olfactory_sensory_neuron_morphology	0.03066
39	MP:0001695_abnormal_gastrulation	0.03151
40	MP:0002461_increased_immunoglobulin_level	0.03227

Table 5.27. Top 31-40 mammalian phenotypes with differentially expressed genes comparing 10 nM BPA treatment to 3 nM treatment in placental explants using MGI Mammalian Phenotype database via Enrichr. Index number 33: impaired glucose tolerance. Glucose metabolism and diabetes were differentially regulated phenotypes/diseases in BPA treated BeWo cells.

Index	Name	P-value
1	Neurobehavioral Manifestations	0.003000
2	Insulin Resistance	0.01507
3	Neoplasms	0.03691
4	Blood Coagulation Factors	0.07830
5	Inflammation	0.06590
6	Personality	0.06320
7	Lipoproteins, HDL	0.1618
8	Leukocyte Count	0.1613
9	Insulin	0.1974
10	Eosinophils	0.2142

Table 5.28. Top 10 diseases with differentially expressed genes comparing 10 nM BPA treatment to 3 nM treatment in placental explants using dbGAP database via Enrichr. Index number 2: insulin resistance. Insulin resistance was differentially regulated in BPA treated BeWo cells.

There were little similarities between BeWo cells and placental explants treated with 3 nM BPA. However, tables 5.17-5.19 show that placental cells treated with 10 nM BPA showed similarities in gene expression to BeWo cells treated with 3 nM and/or 10 nM BPA, such as caveolin signaling, genes related to body fat and circulating LDL levels, as well as diabetes. Interestingly, when 3 nM BPA treated placental explants were compared to 10 nM placental explants, there was a distinct difference in expression of genes involved in the pathogenesis of diabetes and related to body fat across multiple databases and ontologies.

5.3. Discussion

This chapter aimed to examine transcriptional changes in BeWo cells after treatment with BPA. The most significantly enriched terms were found within the 3 nM BPA non-syncytialised BeWo gene set. This data highlights the fact that lower levels of BPA may be more potent in affecting gene expression than higher levels of BPA. One of the possible mechanisms by which this effect may occur is via receptor upregulation at low doses of BPA, which has been documented in our studies as well as in the literature (Liu et al., 2010; Piccart, Parker, & Pritchard, 2003; Vandenberg et al., 2012). As rapid signaling events via membrane-bound estrogen receptors are well-documented in the literature (Marczell et al., 2018; Vandenberg et al., 2012), it is possible that the effects seen in low levels of BPA treated BeWo cells are mediated via an upregulation of ERα.

One of the most significantly upregulated genes in this cohort was CAV1, which is a gene known to be involved in the palmitoylation of ERα, as the protein Caveolin-1 which it transcribes binds to the estrogen receptor and secures it to caveolae/lipid rafts on the cell membrane (Adlanmerini et al., 2014; Chambliss et al., 2000; Levin, 2005), also pointing to the fact that membrane-bound ERα may be at least in part eliciting the response to lower doses of BPA not seen in higher doses. As rapid intracellular responses to receptor signaling may also elicit genomic responses, this is one mechanism of action that could be postulated. However, CAV1 was more significantly upregulated in 10 nM BPA treated BeWo cells than in 3 nM BPA treated cells, therefore more research into this hypothesis is needed.

One of the significantly upregulated genes after treatment with 3 nM BPA was Placenta-specific 1 (PLAC1). PLAC1 is a gene which was first discovered in the placenta, but is also expressed in testes and different types of cancers (Chang et al., 2014; Cocchia et al., 2000; Silva et al., 2007). Although its specific functions are not completely known, it is associated with processes that relate to placental and fetal development (Jackman, Kong, & Fant, 2012) although some studies have been contradictory. For example, Singh et al. found that downregulation of PLAC1 was associated with hyperplastic placentae (Singh et al., 2004), findings which were corroborated (Jackman et al., 2012). However, Suemizu et al. found an association between PLAC1 overexpression and hyperplastic placentae in mice (Suemizu et al., 2003). Other findings which delineate PLAC1 as a gene important for regulating placental development focus on mapping PLAC1 in close proximity to the hybrid placental dysplasia (lhpd) locus, located on the X chromosome, and a paucity of genes in this region suggests a high likelihood of involvement of PLAC1 in placental dysplasia (Hemberger et al., 1999; Massabbal et al., 2005).

In terms of fetal development, the role of PLAC1 has been demonstrated in the literature. PLAC1 downregulation has been associated with fetal large for gestational age (LGA) pregnancies and upregulation of PLAC1 has been associated with less observed frequencies of both LGA and small for gestational age (SGA) pregnancies (Deyssenroth et al., 2017). The regulation of growth during pregnancy not only applies to the fetus, but also to the placenta. PLAC1 has been implicated in placentomegaly

in mice (Suemizu et al., 2003) a condition which has implications in various fetal and placental disorders such as Beckwith-Wiedemann Syndrome (Tunster, Van de Pette, Creeth, Lefebvre, & John, 2018) non-immune hydrops fetalis (Berger et al., 2018) Kagami-Ogata Syndrome (Kagami et al., 2015) and placental mesenchymal dysplasia (Pawoo & Heller, 2014).

The important role of PLAC1 in placental function has been highlighted by studies focusing on other aspects of placental development. One study has found an involvement of PLAC1 in trophoblast differentiation, where knockdown of PLAC1 in mice resulted in the impaired differentiation of trophoblast stem cells into most subpopulations of trophoblast (Gu, Wan, Yao, Peng, & Chang, 2017). In humans, expression of PLAC1 was found to be associated with differentiation of the villous trophoblast, whereby PLAC1 was upregulated during trophoblast differentiation and regulated by growth factors which also play a role during differentiation, such as FGF-7 and EGF (Massabbal et al., 2005). Similarly, downregulation of PLAC1 in primary cytotrophoblasts resulted in less syncytialisation (Chang et al., 2016) again highlighting the role of PLAC1 as a regulator for trophoblast differentiation.

It has now been shown that PLAC1 is upregulated in many different types of cancer, including breast cancer, ovarian cancer, gastric cancer, lung cancer, colorectal and liver cancer (Dong et al., 2008; Koslowski et al., 2009; F. Liu et al., 2014; F. Liu, Shen, Kang, Zhang, & Song, 2015; W. Liu et al., 2012; Xia Wang, Baddoo, & Yin, 2014). As tumours arise due to an increase in cell proliferation, it is important to consider that PLAC1 upregulation might cause an increase in cell proliferation. Studies have found that PLAC1 upregulation causes an increase in phosphorylated AKT (p-AKT), suggesting that downstream effects of PLAC1 are at least in part due to AKT phosphorylation (Koslowski et al., 2009; Wagner et al., 2013; L. Yang et al., 2018). These findings suggest that PLAC1 upregulation could have similar effects in the placenta, causing an upregulation of p-AKT and thereby promoting cell proliferation.

Another upregulated gene after BPA treatment was Leptin. Leptin is an adipokine which is secreted mainly by white adipose tissue in the body and is also expressed in other tissues such as brown adipose tissue, muscle and the placenta (D'souza et al, 2017; Wang et al, 1998; Masuzaki et al, 1997). Leptin levels in humans and rodents

positively correlate with body fat mass, as most of the leptin is secreted by adipose tissue, and 50% of leptin in the circulating blood is correlated with the degree of obesity (Ahren et al, 1997). The main function of leptin is regulation of food intake, whereby leptin functions as an important mediator between adipose tissue and the brain, enabling the regulation of appetite and food intake homeostasis (Zhou & Rui, 2013). Leptin binds to the leptin receptor (LRb), where it leads to the activation of the Jak/STAT and MAPK pathways (Buettner et al, 2013).

The exact function of leptin in the placenta, where it is also secreted, is less well established than it's effects on weight and appetite homeostasis, although recent literature has provided a more detailed picture of placental leptin physiology (Gambino et al, 2012). The expression and secretion of leptin by trophoblasts was demonstrated as early as 1997 (Masuzaki et al, 1997). It has been shown that leptin is a modulator of the endocrine function of the placenta (Coya et al, 2006) due to its various effects on hormones secreted by the placenta such as the increase of basal leptin production by estrogen and increase of HCG production through leptin (Chardonnens et al, 1999), as well as inhibition of progesterone secretion (Cameo et al, 2002) and the inhibition of leptin secretion through human placental lactogen (Coya et al, 2005). Different cytokines, such as IL-1α, IL-1β, IFN-γ and IL-6 also play a role in placental regulation of leptin as well as glucocorticoids, insulin, hypoxia and cAMP (Gambino et al, 2012; Henson & Castracane, 2006; Maymó et al, 2011; Meißner et al, 2003; Fontana et al, 2011; Fontana et al, 2010). Furthermore, leptin is involved in the implantation process of the embryo, by increasing trophoblast matrix metalloproteinase expression, allowing for better cell invasion (Castellucci et al, 2000; Gambino et al, 2011; Chrelias et al, 2016), a mechanism which has been associated with the STAT-3 signalling pathway (Corvinus et al, 2003). Moreover, leptin has been implicated in placental development in terms of angiogenesis and immunomodulation (Gambino et al, 2012; Henson & Castracane, 2006).

Leptin has also been implicated in many pathologies throughout pregnancy and during the later life of the fetus. Numerous studies have shown, for example, that there is a correlation with maternal plasma leptin levels and the development of gestational diabetes (GD) (Fatima, Alam, Chaudhry, & Khan, 2017; Jeon, Hong, & Lee, 2017;

Lobo et al., 2018; Popova et al., 2018; Sweeting et al., 2018). GD is diagnosed with any new-onset glucose intolerance or first manifestation of glucose intolerance during pregnancy (Brink et al, 2016). Because leptin is elevated in obese women, and also during pregnancy, it is difficult to assess the direct correlation between leptin and GD, as obesity itself is a predictor of GD. Studies on the direct correlation between leptin levels and GD have been in disagreement, however one study found that leptin levels were raised in the GD group of pregnant women, even when adjusting for confounders (Fatima et al, 2016).

Because both leptin and obesity in general play a role in inflammatory response and immunomodulation, it is often difficult to assess whether pathologies that are at least partly due to varying immune responses during pregnancy are caused by an increase in leptin levels, an increase in fat mass, or both. Pre-eclampsia is another multifactorial disease which is linked to immune response, obesity, and elevated leptin levels. Pre-eclampsia is defined by a new-onset hypertension and proteinuria during pregnancy (Güngör et al, 2017). Some studies have shown higher serum leptin levels in pregnant women with pre-eclampsia (Güngör et al, 2017; Song et al, 2016, El Shahat et al, 2013; Taylor et al, 2016; Salimi et al, 2014) and most of these studies control for confounders such as BMI. It is however, unknown whether leptin elevation is a consequence of PE or vice-versa, or whether there are other factors causing this correlation.

Another common complication in pregnancy is intrauterine growth restriction (IUGR). IUGR is known as a condition where babies are small for gestational age (SGA), meaning below the 10th percentile at gestational age, a condition usually caused by placental insufficiency (Krishna & Bhat, 2017). Perinatal mortality of fetuses with IUGR is 12 times higher than that of normal gestational weight babies (Monk & Moore, 2014), and it can programme fetuses to develop future metabolic diseases such as early-onset insulin resistance (Dessı et al, 2015). There is an association between higher leptin expression and IUGR (Krishna & Bhat; Nezar et al, 2009). One possible mechanism of action is an increase in TGF-beta via the activation of the JAK-STAT pathway (Krishna & Bhat), possibly triggering apoptosis. Another mechanism by which leptin could act as a mediator towards IUGR is by upregulating endothelin-1 (ET-1) in endothelial cells, as higher ET-1 levels have been related to hypertension and

atherosclerosis (Quehenberger et al, 2002), processes that confirm vascular changes, which can cause placental dysfunction, leading to PE and ultimately IUGR.

Another upregulated gene after BPA treatment was Hif-1α. Hif-1α is a protein associated with hypoxia and regulation of angiogenesis, two factors that play vital roles in the development and physiological functioning of the placenta. When hypoxia reaches less than 5% oxygen, dimerization of Hif-1α and Hif-1β occurs, resulting in the heterodimer HIF-1 which binds to the hypoxia response element (HRE), (Dunwoodie, 2009) and induces expression of genes involved in cell proliferation, angiogenesis, glucose metabolism and apoptosis, among others (Highet et al., 2015; Rocha, 2007). One of the genes upregulated by HIF-1 is vascular endothelial growth factor (VEGF) (Depoix, de Selliers, Hubinont, & Debieve, 2017; G. L. Wang, Jiang, Rue, & Semenza, 1995), a crucial gene regulating angiogenesis of the fetal-placental unit during pregnancy. VEGF binding to its receptor initiates signaling pathways that relate to factors such as cell survival, vascular permeability and the initiation of further signaling pathways such as the MAPK pathway regulating endothelial cell proliferation (Olsson, Dimberg, Kreuger, & Claesson-Welsh, 2006; Sahay et al., 2017). As proteins and signaling pathways related to angiogenesis such as VEGF play a role in common pregnancy complications such as preeclampsia, it is likely that there is a correlation between Hif-1α expression and the development of preeclampsia during pregnancy, and Rajakumar et al. have shown an upregulation of Hif-1α expression in preeclamptic placentae (Rajakumar, Brandon, Daftary, Ness, & Conrad, 2004). It was also shown that Hif-1α regulates TGFβ expression, and a decrease of these factors resulted in increased trophoblast invasion and differentiaton (Caniggia et al., 2000) Another relevant pregnancy complication related to preeclampsia, intrauterine growth restricition (IUGR) (Ness & Sibai, 2006) has been shown by several studies to involve the expression of Hif-1α. In rats, aberrant inflammation was linked to inadequate perfusion of the uterus and placenta, which in turn lead to a decrease in oxygen and accumulation of Hif-1α in IUGR pregnancies (Robb, Cotechini, Allaire, Sperou, & Graham, 2017). In growth restricted-twins, Hif-1α was upregulated compared to non growth-restricted twins suggesting a role of hypoxia in IUGR (G. L. Zhang et al., 2015).

Another significantly upregulated gene in both 3 nM and 10 nM BPA treated non-syncytialised BeWo cells was placenta-specific protein 8 (PLAC8), which was first

discovered as a placenta-enriched gene in a placenta specific microarray screen (Galaviz-Hernandez et al., 2003; Maria Jimenez-Preitner et al., 2011). Since its initial discovery, PLAC8 has been implicated in a multitude of different functions depending on the type of tissue. In hepatocellular carcinoma, downregulation of Plac8 was found to enhance cell proliferation via PI3K/Akt/GSK3β and Wnt/β-catenin signaling (Lei Zou et al., 2016). This pathway was also activated after knockout of PLAC8 in nasopharyngeal carcinoma cells (Yang et al., 2018).

On the other hand, PLAC8 was found to have highly proliferative functions in other tissues. In mouse pancreatic ductal carcinoma (PDAC), PLAC8 was found to be highly upregulated, and inhibition of PLAC8 inhibited cell growth by modifying cyclin D1 (Kaistha et al., 2016). Similarly, the overexpression of PLAC8 lead to increased growth, resistance to apoptosis as well as tumourigenic differentiation and higher levels of phosphorylated Akt1 in fibroblasts (Rogulski et al., 2005) as well as being positively correlated with metastasis and tumour size in clear cell renal cell carcinoma (L. Shi et al., 2017). PLAC8 has been implicated in epithelial-to-mesenchymal transition (EMT) in colon cancer cells, by increasing cell motility and invasiveness (C. Li et al., 2014). Similarly, although the function of PLAC8 in the placenta is still not well elucidated, a recent study has found that PLAC8 is expressed on the fetomaternal interface, where it plays a role in promoting trophoblast invasion and migration. PLAC8 also activates Cdc42 and Rac1 and is significantly upregulated under hypoxic conditions and in preeclamptic placentae (Chang et al., 2018).

Interestingly, PLAC8 has recently been implicated in diseases such as obesity, type 2 diabetes and gestational diabetes. A recent study found that PLAC8 was a strong candidate for playing a role in the onset of type 2 diabetes when associated with obesity, using an obese type 2 diabetic rat model that displays diabetes-related pathologies similar to humans (Sasaki, Kotoh, Watadani, & Matsumoto, 2015). Furthermore, PLAC8 was found to be highly expressed in neonatal cells exposed to GD and expression of PLAC8 was correlated with maternal hyperglycemia (Blue et al., 2015). PLAC8 also plays a role in adipogenesis, brown fat differentiation, and body weight control by controlling C/EBPβ expression (Jimenez-Preitner, Berney, & Thorens, 2012; Jimenez-Preitner et al., 2011).

Another significantly upregulated gene in both 3 nM and 10 nM non-syncytialised BeWo cells was caveolin-1 (CAV1). CAV1 is a protein that is found in caveolae, which are 50 to 100 nm wide invaginations of the cell lipid bilayer. The function of CAV1in the placenta has not been fully elucidated, but it has been implicated in the transport of lipids, glucose homeostasis control, regulation of cell signalling, and membrane trafficking (Asterholm, Mundy, Weng, Anderson, & Scherer, 2012; Ding et al., 2017; Fernández-Rojo et al., 2013; M. Li et al., 2017; Parton & Simons, 2007; Smart et al., 1999).

CAV1 has been implicated in the regulation of actin remodelling and cell migration, as well as cell invasion via stimulation of the Caveolin-1/p85α/Rab5/Tiam1/Rac1 signaling axis in 3 different metastatic cell lines in mouse and human (Díaz, Mendoza, Silva, Quest, & Torres, 2014). CAV1 also has been found to play a role in cell proliferation, as it is upregulated after stimulation with E2 in breast cancer cells, where it inhibits apoptosis and promotes autophagy (Nah et al., 2017; Panepistēmio tēs Krētēs. et al., 2014). CAV1 Was also reported to increase proliferation in osteoblasts via activation through leptin and subsequent Akt phosphorylation, resulting in a decrease of apoptosis in these cells as well as an increased proportion of cells in S-phase (Lin Zou et al., 2016). On the other hand, CAV1 has been reported to decrease growth in breast cancer cells and have tumour-suppressor properties (Shi et al., 2016). Another function of CAV1 is to regulate water and salt absorption, as CAV1 in renal cells promoted the reabsorption of water and salts and is involved in the upregulation of aquaporin 4 in astrocytes (Bi et al., 2017; Willière et al., 2018).

In pregnancy, CAV1 has been found to decrease but not disappear with formation of syncytium (Levi et al., 2016; Linton, Rodriguez-Linares, Rashid-Doubell, Ferguson, & Redman, 2003). CAV1 has been shown to be associated with glucose and fatty acid transport in the placenta by inducing AMPK and reducing the GLUT1 signaling pathway, involved in reversing macrosomia due to gestational diabetes (Yao et al., 2017). Furthermore, CAV1 has been implicated in the mechanism of edema in pre-eclampsia following hypoxia of trophoblasts through the HMGB1/TLR4/CAV-1 pathway (Jiang et al., 2014).

In both 3 nM and 10 nM BPA treated syncytialised cells, SIM2 was significantly upregulated. SIM2 encodes for a protein that was discovered to be vital for CNS midline development in Drosophila (Crews, Thomas, & Goodman, 1988; Fan et al., 1996; Muenke et al., 1995) as well as murine SIM2 playing a role in directional development of other organs such as the gut and of muscle development (Crews et al., 1988; Fan et al., 1996; Lewis & Crews, 1994). As a basic-helix-loop-helix (bHLH) protein, it belongs to a group of transcription factors which play an important role in cell fates as well as differentiation and proliferation in embryo development (Fan et al., 1996; Jan & Jan, 1993).

As a gene that is found on chromosome 21 q22.2-q22.3, SIM2 was discovered to be in the "Down syndrome critical region" (Muenke et al., 1995; Peterson et al., 1994) and to play a role in Down syndrome development, with overexpression of SIM2 potentially being a causal factor in the pathogenesis of Down syndrome in humans (Chrast et al., 1997; Fan et al., 1996; Muenke et al., 1995; Rachidi et al., 2005; Vialard et al., 2000). Furthermore, SIM2 has been shown to be upregulated in certain types of tumours such as pancreatic cancer (DeYoung, Tress, & Narayanan, 2003; Farrall & Whitelaw, 2009) and prostate cancer (Arredouani et al., 2009; Farrall & Whitelaw, 2009; Lu, Asara, Sanda, & Arredouani, 2011), and to have anti-apoptotic effects via an inhibitive effect on Hif1-α (Farrall & Whitelaw, 2009; Nakamura et al., 2017). Interestingly, SIM2 plays a role in mediating the neurotoxic effects of hyperglycaemia in the diabetic rat (Xiaolan Wang et al., 2013), although these findings have not been tested in other tissues or organs.

Another upregulated gene in 3 nM and 10 nM BPA treated syncytialised BeWo cells is cytoplasmic linker-associated protein 2 (CLASP2), which is a gene belonging to the CLASP family of microtubule associated proteins that bind to microtubules and are involved in the stabilization of microtubules (Akhmanova et al., 2001) and regulates the function of the spindle and kinetochore during mitosis (Pereira et al., 2006). It has also recently been found to be a protein involved in epithelial-to-mesenchymal transition (EMT) as well as bladder cancer progression (Zhu et al., 2017) as well as being important for cytoskeletal arrangement during neocortical development (Dillon et al., 2017). Interestingly, a recent study has found a role for CLASP2 in the direction

of GLUT4 to the cell surface after phosphorylation as a response to an increase in insulin (Langlais et al., 2012), making CLASP2 another gene which is upregulated by BPA which plays a role in insulin signaling.

The most enriched molecular function within this gene set was guanyl-nucleotide exchange factor (GEF) activity, and the most enriched biological pathways were Rac1-signaling and RhoA signaling. RhoA and Rac1 are types of GTPase, which are a family of signaling proteins belonging to the Ras GTPase superfamily (Zandvakili, Lin, Morris, & Zheng, 2017). These molecular switches are either bound to GTP (active state) or GDP (inactive state) and when active, generate a cellular response until GTP is hydrolysed to GDP (Etienne-Manneville & Hall, 2002). Guanine nucleotide exchange factors (GEFs) catalyse loading of GTP onto GTPases and thereby activate GTPase signaling (Zandvakili et al., 2017).

Rho GTPase and Rac have a multitude of functions within the cell, one of which is contributing to the regulation of G1 cell cycle progression, by promoting G1 entry and progression into S phase (Etienne-Manneville & Hall, 2002; Olson, Ashworth, & Hall, 1995). Rho inhibits expression of the expression of p21, which in turn inhibits cyclin/Cdk as well as inducing cyclin D1 expression during mid-G1 via activation of extracellular-signal-regulated kinase (ERK/MAPK) thereby promoting proliferation (Etienne-Manneville & Hall, 2002; Olson et al., 1995; Olson, Paterson, & Marshall, 1998) whereas Rac stimulates the c-Jun kinase JNK/SAPK (Olson et al., 1995). One of the main ways that RhoA is activated is through G protein-coupled receptors (GPCRs), and it has been well established that GPCR ligands such as thrombin, lysophosphatidic acid and sphingosine-1 phosphate activate RhoA (Ishii et al., 2001; Moers et al., 2003; Nobes, Hawkins, Stephens, & Hall, 1995; Post et al., 1996; Walsh et al., 2008; Yu & Brown, 2015). Rac1 is activated by different stimuli, such as growth factors, tyrosine-kinase receptors, as well as ligands that bind to G-protein-coupled receptors such as the ER GPR30 (Li et al., 2016; Wertheimer et al., 2012). A study has also found that treatment of hippocampal cells with BPA for 24 hours upregulated the expression of Rac1/Cdc42, demonstrating the involvement of BPA in regulating this pathway after 24 hours of exposure (Xu et al., 2014).

In 3 nM BPA treated non-syncytialised BeWo cells, the NCI Nature 2016 database showed that insulin resistance was a highly enriched pathway (p= 0.01312). Insulin signaling was also highly enriched using the WikiPathways 2016 database (p= 0.03006), as well as leptin insulin overlap in the mouse, differentiation of white and brown adipose tissue (p= 0.01474) and leptin signaling pathway (p= 0.03426). Regulation of RhoA activity was also highly significantly enriched using the KEGG 2016 database (p=0.0003159). Interestingly, these significantly enriched pathways have shown to be linked in a multitude of studies. Insulin has been shown to affect ROCK1 signaling (Kong et al., 2014) and insulin resistance has been found to be mediated via modulation of the RhoA signaling pathway in mice (Tao et al., 2015) and pancreatic β-cells grown in 3D (Liu et al., 2014). Furthermore, patients with metabolic syndrome have been found to show higher levels of RhoA/Rho-kinase activity (Leguina-Ruzzi et al., 2015).

Leptin is an adipokine which is secreted by fat cells as well as the placenta (Masuzaki et al., 1997), and among its other functions, increases insulin secretion of pancreatic β-cells and contributes to insulin resistance (Lee et al., 2001; Spiegelman & Flier, 2001). Together, insulin and leptin control glucose metabolism, and play a role during pregnancy by regulating fetal growth and development, with leptin levels in umbilical cord blood positively correlating with neonate fat mass and body weight (Guzmán-Bárcenas et al., 2016). Studies have shown that increased RhoA/Rho kinase signaling mediates skeletal muscle insulin resistance (Chun et al., 2011; Tao et al., 2015) and that the RhoA/ROCK pathway is involved in insulin release in mouse pancreatic β-cells (Liu et al., 2014). Furthermore, multiple studies have shown that leptin can activate the RhoA pathway or its effector ROCK1 to induce different processes such as cell invasion, reorganization of the actin cytoskeleton, homeostasis of feeding behaviour and increasing colonic tight junction permeability (Ghasemi, Hashemy, Aghaei, & Panjehpour, 2017; Huang et al., 2012; Le Dréan et al., 2014) and that RhoA signaling is involved in the processes by which leptin promotes hypertension, ROS generation and atherosclerosis (Ghantous et al., 2015).

Rac1 has also been implicated in insulin signaling. It has been well established that insulin and the insulin receptor activate Rac1 in skeletal muscle, thereby increasing

traffic of the glucose transport protein GLUT4 and that Rac activation is impaired in insulin resistance (Chiu, Jensen, Sylow, Richter, & Klip, 2011; JeBailey et al., 2007; Ueda et al., 2010; Ueda, Kataoka, & Satoh, 2008). It has also been shown that Rac1 is activated by insulin in mouse adipose tissue (Takenaka, Nihata, Ueda, & Satoh, 2017). Furthermore, Rac1 has been implicated in excess ROS accumulation under diabetic conditions (Sidarala & Kowluru, 2017), implying a role for Rac1 in chronic low levels of inflammation, one of the hallmarks of obesity related disorders and metabolic syndrome in general, which includes type 2 diabetes (Zhou et al., 2015).

Finally, we used microarray to analyse differentially expressed genes in placental explants, in order to discern effects of 3 nM and 10 nM BPA on these tissues and elucidate whether gene expression results were similar when compared to BeWo cell, both syncytialised and non-syncytialised. Three siginificantly upregulated genes in this tissue in both 3 nM and 10 nM treated explants were interleukin 17F (IL-17F), epithelial cadherin (e-cadherin) and resistin, which play a role in placental physiology.

IL-17F is a pro-inflammatory cytokine that is primarily expressed by T-cells and also produced by other immune cells such as monocytes and neutrophils (Pongcharoen et al., 2007). Recently, the gene and protein expression of IL-17F has been found in human normal term placenta trophoblast (Pongcharoen et al., 2007). IL-17F is produced by Th17 T-cells that play a major role in inflammation, and IL-17F secreted by these cells is involved in the pathogenesis of a multitude of inflammatory diseases (Sandquist & Kolls, 2018), such as rheumatoid arthritis (Chabaud et al., 1999), psoriasis (Krueger et al., 2007), and asthma (Bullens et al., 2006). IL-17F binds to the IL-17 receptor (IL-17R) which is expressed on most cells in the human body (Miossec & Kolls, 2012). One of the main pathways by which the binding of IL-17 to IL-17R mediates its effects is by activation of the NF-κB pathway (Miossec & Kolls, 2012) and thereby provokes an immune response and inflammation by mediation of a multitude of different factors (Sun, 2011).

Studies have investigated the effects of IL-17 on the placenta. As IL-17 is a pro-inflammatory cytokine, these effects can be destructive on the placental level and therefore cause placental pathologies that influence the pregnancy. One such pathology is pre-eclampsia (PE). Studies have found that increased levels of IL-17 in

the placenta are linked to fetal growth restriction and PE in the human placenta (Cornelius & Lamarca, 2014; Darmochwal-Kolarz et al., 2017) as well as low fetal weight in mice with malaria (Fitri et al., 2015). Placental oxidative stress mediated by IL-17 was linked to hypertension in pregnancy (Dhillion et al., 2012), another determinant of pre-eclampsia. Furthermore, another study found that diabetic mothers had increased levels of IL-17 in the placenta, speaking for a role of IL-17 in the inflammatory environment produced by hyperglycemia (Hara et al., 2016).

E-Cadherin is a type of cadherin. Cadherins are proteins that mediate cell-cell adhesion and play a role in other biological processes such as cellular signaling, cell division, inhibition of apoptosis, embryo implantation, differentiation, migration, and suppression of tumour invasion (Alahari, Reddig, & Juliano, 2002; Christofori & Semb, 1999; Kokkinos, Murthi, Wafai, Thompson, & Newgreen, 2010; Perez-Moreno, Jamora, & Fuchs, 2003; Ranscht, 1994; Yap & Kovacs, 2003). In the placenta and in BeWo cells, E-Cadherin is a marker of syncytialisation, as syncytialised cells lose E-Cadherin as the cell membrane breaks down to from a syncytium (Coutifaris et al., 1991).

E-Cadherin has been implicated in the pathogenesis of PE, where it was found to be upregulated in human syncytiotrophoblast in patients with PE (Li et al., 2014). Other studies found that E-cadherin upregulation led to a decrease in trophoblast invasion, therefore leading to shallow placentation in PE (Zhang et al., 2013), and that non-fused E-cadherin positive cells were more common in PE placentae and placentae in patients with gestational hypertension, (Al-Nasiry, Vercruysse, Hanssens, Luyten, & Pijnenborg, 2009; Brown, Lacey, Baker, & Crocker, 2005; Li, Cheung, Tsao, Cheung, & O, 2003) another risk factor for the development of PE.

Another upregulated gene in placental explants treated with BPA for 24 hours was resistin. Resistin is a protein secreted from white adipose tissue in rodents, and macrophages in humans (Schwartz & Lazar, 2011; Steppan et al., 2001), that was found to also be expressed by the placenta (Yura et al., 2003). Resistin (‘resistance to insulin’) gets its name from the fact that it has the ability to interfere with insulin metabolism and is linked to obesity and diabetes (Mostafazadeh, Haiaty, Rastqar, & Keshvari, 2018; Steppan et al., 2001). The release of resistin is stimulated by

inflammatory agents such as TNF-α (Schwartz & Lazar, 2011), and is itself pro-inflammatory (Mostafazadeh et al., 2018).

In the placenta, resistin expression has been found to be upregulated in the human placenta in mothers with PE (Erol et al., 2016), and serum resistin levels were also found to be higher in mothers with PE (Seol et al., 2010). Resistin has also been linked to gestational diabetes in pregnancy. Resistin was found to increase placental glucose intake (Di Simone et al., 2009), and resistin protein levels were associated with insulin levels, BMI, and glucose levels in pregnancy (Zhou et al., 2006). Furthermore, umbilical serum resistin levels and maternal serum resistin levels were found to be negatively correlated with neonatal birth weight (Cho et al., 2006).

Taken together, these findings highlight the capacity of BPA to affect the BeWo cell genome. The most significant changes were seen in cells that appeared to be most susceptible to BPA treatment, i.e. 3 nM treated non-syncytialised BeWo cells. These changes imply a role of BPA in influencing the metabolism as well as proliferation of placental cells, factors that could significantly affect fetal and placental development as well as determine the outcome of the pregnancy itself. Insulin signaling is a pathway which has been demonstrated to play a major role during pregnancy, as GD, for example, is a disease which can have severe effects on the fetus, pregnancy, and long-term effects on both mother and child, including fetal macrosomia, maternal preeclampsia, neonatal hyperglycemia and respiratory distress syndrome, the development of type 2 diabetes of the mother after pregnancy, as well as an increased risk of the development of obesity and abnormal glucose metabolism of the offspring whether in childhood, adolescence or adulthood (Gilmartin, Ural, & Repke, 2008; Petry, 2018; Schmidt et al., 2001) . As obesity is one of the risk factors of pregnant women for developing gestational diabetes, high leptin levels may also be a contributing factor when it comes to the aetiology of GD. In fact, recent studies have shown the relationship between higher placental leptin and the development of insulin resistance and GD, as well as showing that levels of leptin in the placenta were higher in macrosomic offspring than in normal weight offspring (Shang, Dong, & Hou, 2018; Tsiotra et al., 2018).

When comparing placental explant data to BeWo data, there were vast differences in gene expression. These differences were to be expected, as placental explants do not contain solely trophoblasts, but other cells such as immune cells and vascular cells. Interestingly, however, there were distinct patterns to be seen in differential gene expression when comparing these two different platforms. Especially in the case of diabetes, and factors that could be considered risk factors for the development of diabetes, such as hyperglycemia, both placental explants as well as BeWo cells treated with BPA showed profound gene expression changes in association to the disease, both in single gene expression levels as well as over databases that analyse gene sets. These “pro-diabetogenic” effects of BPA on placental explants were seen solely in explants treated with 10 nM BPA, suggesting that higher levels of BPA, although still relatively low, are needed to affect actual human placenta when compared to BeWo cells, where these effects were seen in both 3 nM and 10 nM treated samples. In conclusion, there is evidence to support the hypothesis that BPA plays a role in the development of metabolic diseases, especially diabetes, during pregnancy mediated through the placenta, which can have profound effects on the mother and the fetus, as well as potentially influencing the future health and development of both.

Chapter 6

From 2Ds to 3Ds and placental explants

6.1 Introduction

As mentioned, the human placenta is vital to the viability of the pregnancy for almost 9 months, controls flow of nutrients and waste products from mother to fetus, and is an endocrine organ which takes part in regulating hormonal homeostasis of both the mother and the fetus. As such, *in vivo* experiments on human placenta are ethically and logistically difficult, and cell lines have played a major role in compensating for lack of tissue availability. Not only are they easier to access than placental tissue, but cell lines can be cultured for weeks, making longer experiments possible, whereas tissue from human placenta typically starts to degrade after 48 hours (Di Santo, Malek, Sager, Andres, & Schneider, 2003). Although cell lines are essential in providing a medium for various placental experiments, more physiologically representative long-term platforms for the testing of placental function are needed, especially as the placenta is one of the least understood -in an endocrine sense-, yet most vital organs (Guttmacher et al., 2014).

Emerging studies suggest that 3D cell cultures can be superior when it comes to accurately representing in *vivo* tissue and organ physiology, as 3D cultures have the capacity to regain intrinsic qualities and mimic the in *vivo* environment, as well as retaining more physiological tissue architecture and cell contacts (Dolznig et al., 2011; Fischbach et al., 2009; Pickl & Ries, 2009; Riedl et al., 2017). Studies have also shown gene expression profiles resemble the actual tissue more in 3D cultured cells when compared to 2D cultured cells, such as colon cancer cell lines (Riedl et al., 2017; Takagi et al., 2007). Using 3D cell culture as an alternative to 2D cell culture might also partially replace animal models, especially when considering the placenta. Animal models never fully mimic any human in *vivo* organ, however the placenta is especially heterogeneous among different types of species, with many aspects, such as high level of invasiveness of the human trophoblast as well as duration of placentation being unique to humans (Carter, 2007; Orendi et al., 2011). Furthermore, there is an ethical rationale behind trying to replace animal models with human-derived models

of the placenta, and therefore it is always necessary to try and devise new methods that find a way around animal laboratory testing and towards techniques that incorporate both high ethical and scientific standards.

As explants from placental tissue are difficult to culture in *vitro* over longer periods of time, one of the goals of this chapter was to find a method which combines the benefit of using actual human tissue as a testing platform as well as the long-term viability characteristics of cell lines. In order to gain further insights into the field of placenta research, especially with the focus on EDC testing, robust models that are human specific, tissue specific and viable for extended periods of time as opposed to 48 hours are needed. To date, placental tissue is often cultured in plastic wells surrounded in media for a couple of days (Douglas, VandeVoort, Kumar, Chang, & Golos, 2009; Sato, Ward, Astern, Kendal-Wright, & Collier, 2015; Steinberg & Robins, 2016). To our knowledge, there are no studies that have investigated the effects on viability on term placental tissue when tissue is cultured in a 3D environment, exposed to low levels (8%) of oxygen as opposed to environmental levels, and surrounded in a hydrogel.

The main aim of this chapter is to initiate the development of tissue culture platforms that represent in *vivo* placenta more accurately as more physiologically relevant models are needed. We have tested methods using BeWo cell grown in 3D as well as placental explants, therefore this chapter has been structured into 2 main aims:

1. Grow BeWo cells on a 3D scaffold and elucidate cellular changes occurring on a morphological, ER expression and hormone expression level.

2. Grow placental explants on a hydrogel scaffold in an attempt to prolong viability for more than the normal 72 hours and test viability by observing morphology and β-hCG levels.

6.2 Results

6.2.1 Platforms for growing BeWo cells in 3D

In order to grow cells on a 3D scaffold, different platforms were tested, as it was necessary to investigate whether BeWo cells grow in a 3D environment, and whether they form physiological structures resembling organoids. Matrigel™ is a common scaffold for 3D cell culture and was therefore employed.

Initially, Matrigel™ was used as a scaffold for cell growth. It is an extracellular matrix-like protein mixture that is secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells (Hughes, Postovit, & Lajoie, 2010) and has been used successfully to grow cell lines in 3D. Due to its nature and dependancy on mouse sarcoma cell production, there was a vast batch variation within different batches. Furthermore, BeWo cells grew in an erratic fashion, with projections that did not resemble placental tissue (Figure 6.1 and 6.2). Due to these concerns, we used a different platform called Growdex® (Figure 6.3-6.7.), which does not have batch variation and is composed of nanofibrillar cellulose derived from birch trees (Bhattacharya et al., 2012).

6.2.1.1 Matrigel™

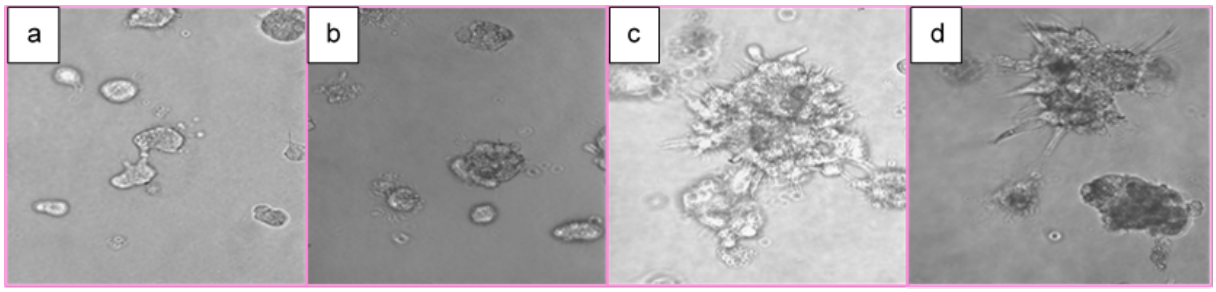

Figure 6.1. Brightfield images of 3D BeWo cells grown in Matrigel™ at different stages of growth. Cells were seeded at 20,000 cells/ml. Row of cells imaged at 5 (a), 8(b), 11(c) and 14(d) days respectively.

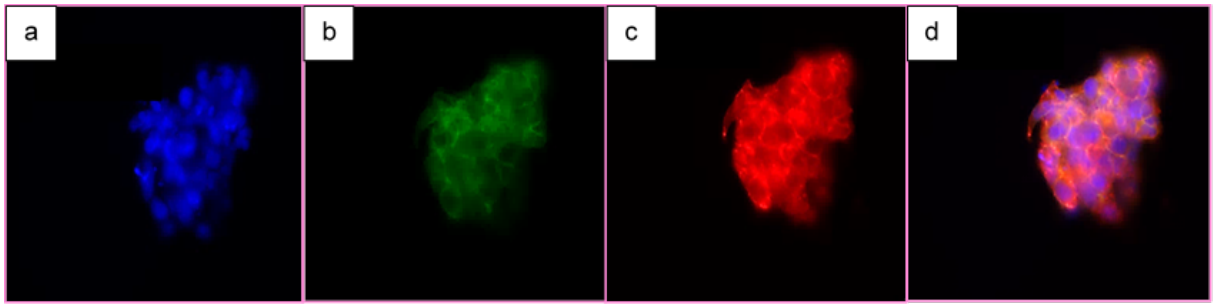

Figure 6.2. Immunofluorescent images of 3D BeWo cells grown on Matrigel™. Blue: DAPI nuclear stain (a), green: e-cadherin (b), red: phalloidin (c), merge (d).

6.1.1.2 Growdex®

Growdex® was used as an alternative to Matrigel™ due to there being no batch variation as well as the fact that the scaffold itself only contains cellulose, thereby having less intrinsic protein function that could interfere with cellular function. Cells were seeded at 80,000 cells/ml and initially grown for a length of 21 days (Figure 6.3). With this scaffold, cells appeared to grow in a more physiological fashion, without the projections that were seen when grown on Matrigel™. Furthermore, cells appeared to grow in clusters and formed round, multinucleated structures (Figure 6.4 and 6.5). These structures physiologically resembled syncytiotrophoblast, the endocrine active differentiated cytotrophoblasts.

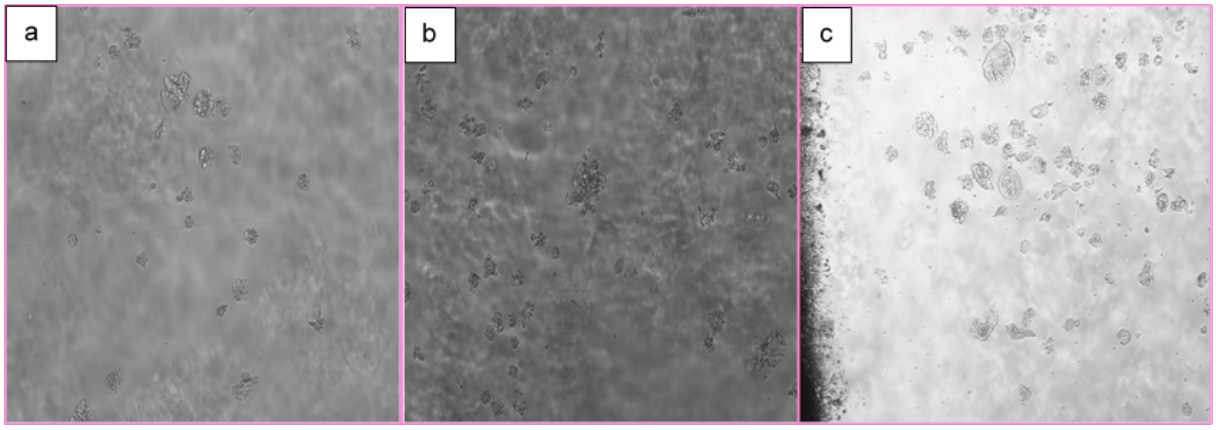

Figure 6.3. Brightfield images of 3D BeWo cells grown in Growdex at different stages of growth. Cells were seeded at 80,000 cells/ml. Row of cells imaged at 6(a) 12(b) and 21(c) days respectively. Cells appear to grow in clusters and have a different, more rounded shape than when grown in Matrigel™.

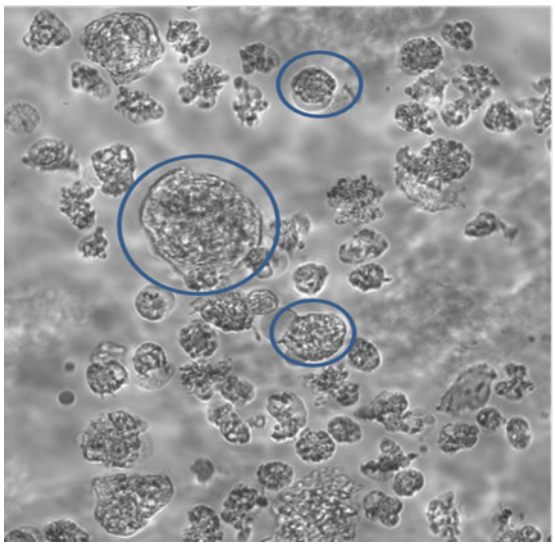

Figure 6.4. BeWo cells grown in Growdex® for 7 days. Cells form rounded multinucleated structures that resemble placental tissue in *vivo* compared to cells grown in 2D (blue circles).

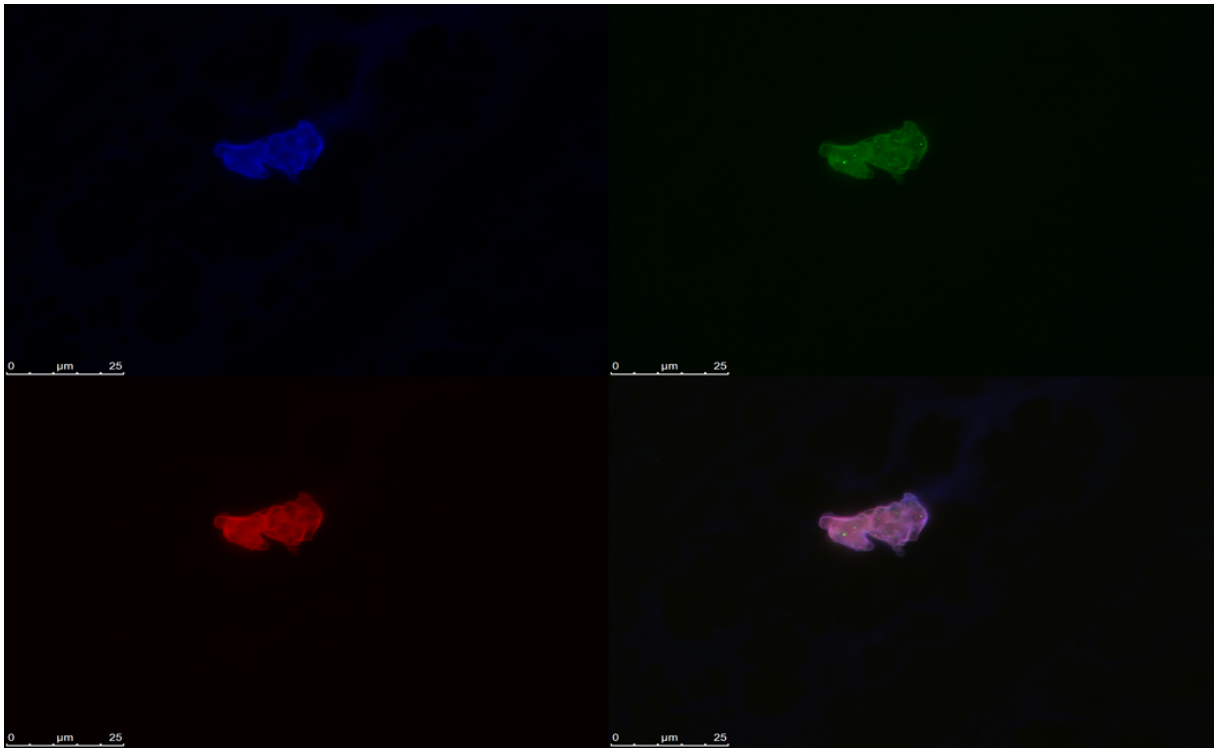

Figure 6.5 Immunofluorescent images of BeWo cells grown in Growdex® for 7 days. Blue: DAPI nuclear stain, green: e-cadherin, red: phalloidin, last panel: merged image.

6.2.2. Gene expression of BeWo cells grown in Growdex® in comparison to cells grown in 2D

In order to assess whether BeWo cells grown in 3D showed different gene expression than cells grown planar tissue culture (2D), we compared ER expression between non-syncytialised BeWo cells grown in 2D and grown in Growdex® (Figure 6.6). Due to previous data suggesting that ERβ expression in BeWo cells was very low, expression levels of ERα and GPR30 only were measured. There was a marked increase in gene expression of ERα in cells grown in 3D (RQ = 0.3) compared to 2D cells (RQ = 0.03). Gene expression of GPR30 also showed an increase in cells grown in 3D (RQ = 0.22) compared to cells grown in 2D (RQ = 0.08).

Due to the impression that BeWo cells grown in Growdex® formed round, multinucleated structures similar to syncytiotrophoblast (Figure 6.4), we compared gene expression levels of the marker of syncytialisation syncytin-2 in BeWo cells grown in Growdex™ and in 2D (Figure 6.7). Cells grown in Growdex™ showed an increase in gene expression of syncytin 2 (RQ = 0.35) compared to cells grown in 2D (RQ = 0.03).

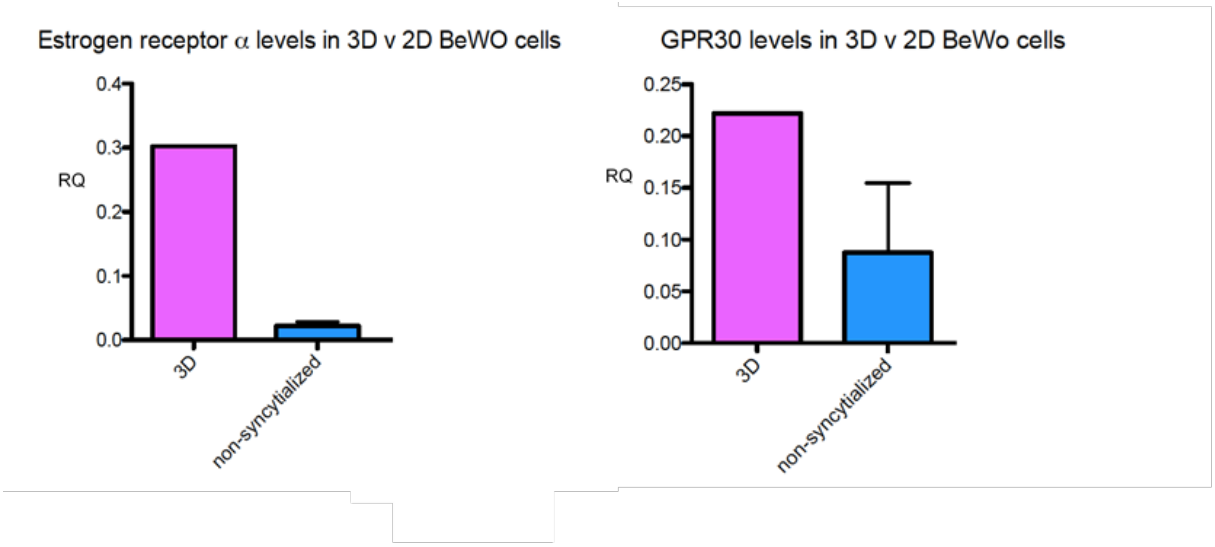

Figure 6.6. Relative expression of estrogen receptors in non-syncytialised BeWo cells grown in 3D (Growdex®) versus non-syncytialised BeWo cells grown in 2D. There is a marked increase in ERα expression in BeWo cells grown in 3D (RQ = 0.3) compared to expression in cells grown in 3D (RQ = 0.03). There is also an increase GPR30 gene expression in cells grown in 3D (RQ = 0.22) compared to cells grown in 2D (RQ = 0.08). ERβ not measured due to lack of ERβ in 2D BeWo cells.

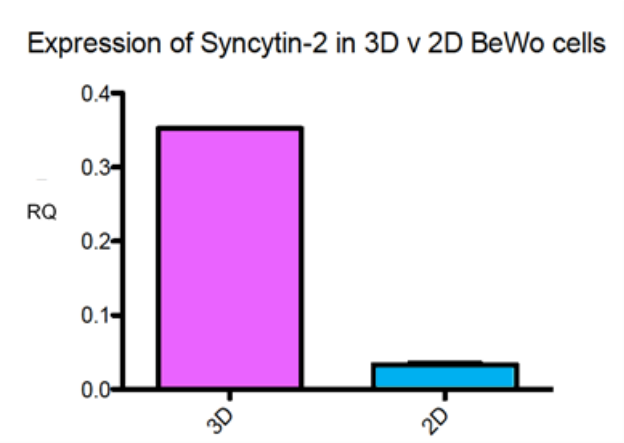

Figure 6.7. Relative expression of syncytin-2 in non-syncytialised BeWo cells grown for 7 days in 3D (Growdex®) versus non-syncytialised BeWo cells grown in 2D. There is a marked increase in syncytin-2 expression in BeWo cells grown in Growdex™ (RQ = 0.35) compared to cells grown in 2D (RQ = 0.03).

6.2.3. Hormone secretion of BeWo cells grown in 3D (Growdex®) in comparison to cells grown in 2D

In order to further assess whether there is a functional change in BeWo cells grown on the 3D platform Growdex™, β-hCG and E2 secretion in these cells was measured (Figure 6.8) and compared to expression profiles of the same hormones during human pregnancy. When measured over 20 days, hormone expression of BeWo cells grown in 3D showed a similar expression profile of β-hCG and E2 over three trimesters of pregnancy.

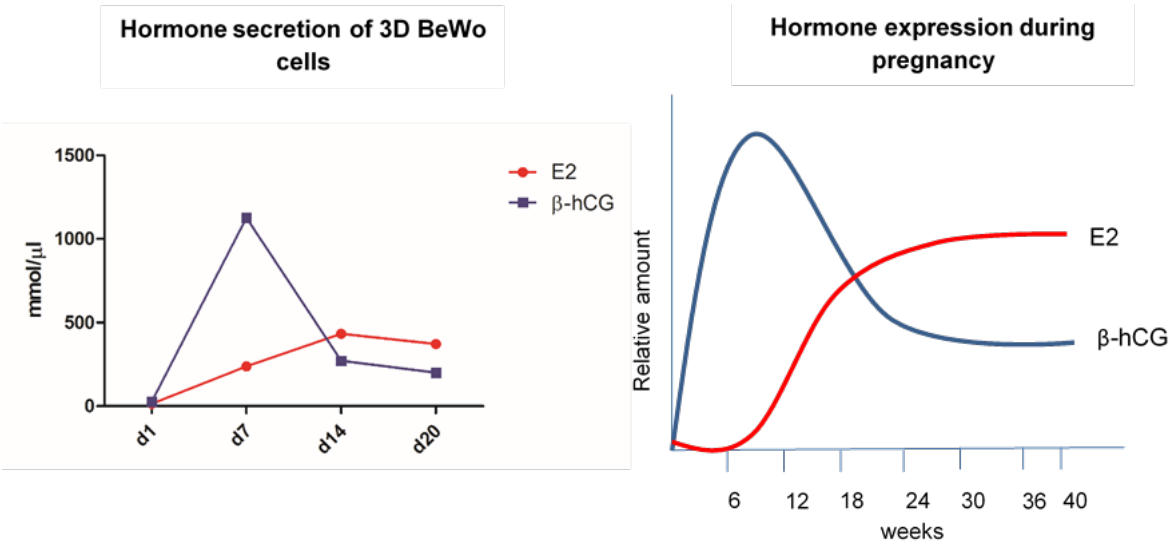

Figure 6.8. Hormone secretion of 3D BeWo cells grown in Growdex® for 20 days compared to hormone expression during pregnancy. Expression profiles of β-hCG and E2 are similar in these cells to expression progiles in blood during human pregnancy. D1-d20: days.

6.2.4. Placental explants grown in GrowDex®

In this part of the study, we have attempted to prolong placental tissue viability and minimize tissue degradation by setting up term placental explant cultures in a novel system. Using GrowDex® hydrogel, a plant-based hydrogel matrix containing cellulose, we have embedded placental explant pieces of 2mm in size between the hydrogel in 96-well plates and covered these cultures in media. Doing this, we have provided an environment for the tissue that structurally is more similar to the extracellular matrix found in *vivo* when compared to the plastic and media which usually surrounds the placental explant, as well as attempting to keep the explants in a low oxygen environment, being embedded within the hydrogel, a characteristic which has been shown to be crucial for placental tissue viability, as atmospheric oxygen levels can affect placental explants and increase apoptosis and inflammation (Brew & Sullivan, 2017).

In order to achieve placental tissue culture lasting over more than 48 hours, placental tissue was embedded into GrowDex® hydrogel with media changed every 5 days. 2mm biopsies were placed in between 2 layers of GrowDex® as previously described.

Tissues were imaged at 9 days and 16 days. Viability of tissues was evaluated by measurement of β-hCG.

Placental explants were grown for 16 days in Growdex™ and imaged at 9 days (Figure 6.9) and at 16 days (Figure 6.10). At 9 days, some images showed intact placental brush-border, an indicator that the tissue is still viable as there is no breakdown of this fragile placental structure. Figure 6.10 shows that at 16 days of growth some explants have also still ratined this brush border, showing potential for explants to be grown for over 2 weeks. There were no other major structural changes between explants at 9 days and explants at 16 days.

To further affirm that explants grown for 16 days embedded in hydrogel were viable, measurements of β-hCG, a hormonal marker of functioning placental tissue were measured at day 6 and day 16 (Figure 6.11). The data shows a 20-fold increase from day 1 to day 6 of 1050 mmol/μl from 55.42 mmol/μl, and a 1-fold decrease to 504.8 mmol/μl on day 16, indicating some loss of viable sample by this time.

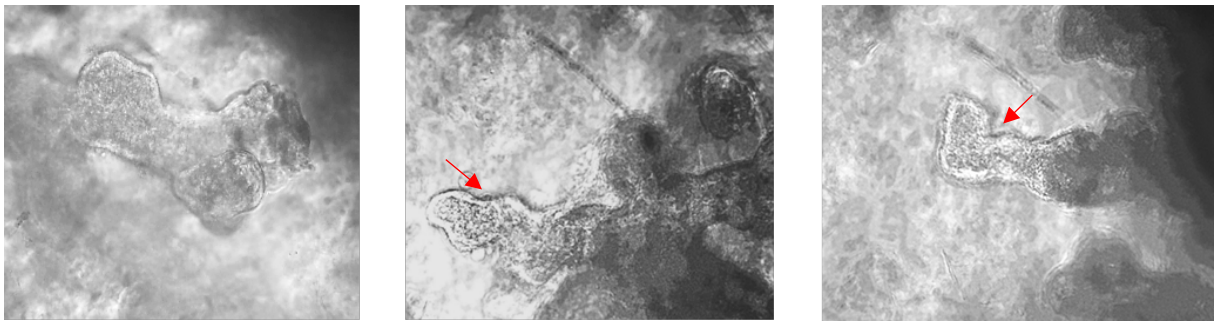

Figure 6.9. Placental explants grown in GrowDex® after 9 days. Red arrows indicate intact placental brush-border giving the impression of viable placental tissue.

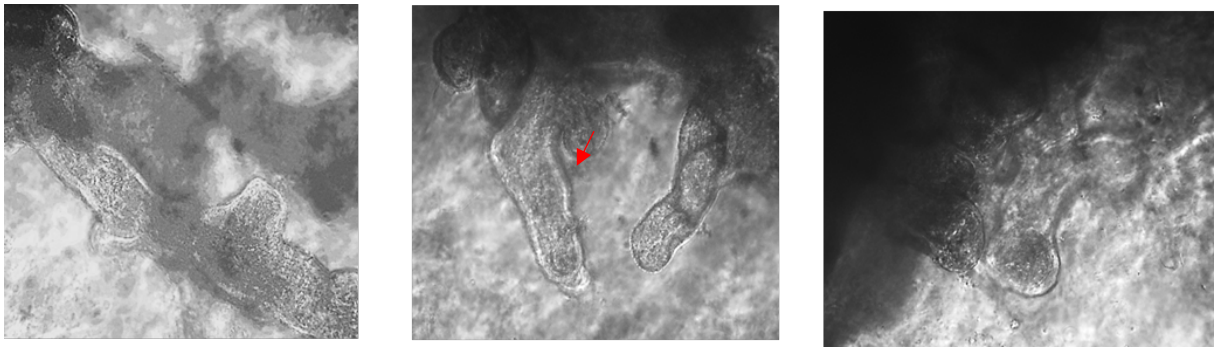

Figure 6.10. Placental explants grown in GrowDex® after 16 days. There do not appear to be major structural differences between the two time-points (compared to 9 days, figure 6.9). Red arrows indicate intact placental brush border giving the impression of viable placental tissue.

β-hCG Expression of explants cultured in Growdex®

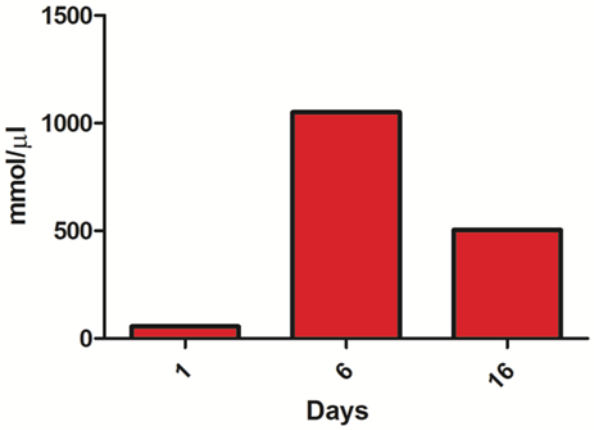

Figure 6.11. β-hCG levels were measured in placental explants embedded in hydrogel on day 1 (55.42 mmol/µl), day 6 (1,050 mmol/µl) and day 16 (504.8 mmol/µl). There was a 50-fold increase of β-hCG secretion in explants at day 6 when explants were embedded into hydrogel, which fell down to 504.8 mmol/µl by day 16. Day one was measured before embedding into hydrogel.

6.3 Discussion

Developing a relevant model of the human placenta is important due to a need for replacement of animal models and the importance of the placenta to not only the viability of the fetus during pregnancy but also the health of the offspring later in life. As a transient organ that is difficult to research and is therefore one of the least studied organs of the human body, valid platforms to test chemicals that can affect mother and child are desperately needed. As EDCs are ubiquitous chemicals that most humans

are exposed to, these substances are especially important to test on the placenta. In this chapter, we have attempted to move from the basic human placental model of cell lines grown in 2D to models that represent the human placenta more accurately.

By growing BeWo cell lines on a GrowDex® hydrogel scaffold, cells were enabled to grow in a more physiological 3D form with more cell-to-cell interactions and resembling organoids. Studies with different cells and cell lines have shown that using a nanofibrillar cellulose hydrogel such as GrowDex® can induce physiological cell traits that are not shown in a 2D setting. In liver progenitor cells, using nanofibrillar cellulose induced cell polarity as well as forming physiological liver structures, and showed expression of typical hepatobiliary drug transporters and mRNA of hepatocyte markers (Bhattacharya et al., 2012; Malinen et al., 2014). Furthermore, nanofibrillar cellulose hydrogel facilitated the differentiation of human pluripotent stem cells (hPSCs) and induced formation of 3D spheroids (Lou et al., 2014). GrowDex® is a reliable scaffold as it is free of any growth factors or proteins, with solely the cellulose concentration determining the properties of the scaffold, such as viscosity. By keeping the cellulose concentration at 1%, the properties of GrowDex® were reproducible, and not subject to any batch variability. Our study is the first study to investigate the effects of growing the BeWo cell line in a nanofibrillar cellulose hydrogel.

In our study we found that BeWo cells grown in a 3D environment using GrowDex® form spheroids not previously seen in 2D culture. These spheroids appear to consist of cells that have merged to form a syncytium, with a shared outer membrane, similar to *in vivo* placental tertiary villi, rather than just forming clusters of cells. In order to confirm that BeWo cells grown in GrowDex® spontaneously syncytialise without the addition of 8-Br-cAMP which is usually needed for syncytialisation in cells BeWo cells grown in 2D, we investigated whether Syncytin-2, a marker of trophoblast syncytialisation (Vargas et al., 2009), is upregulated in BeWo cells grown in GrowDex®. In our experiment comparing non-syncytialised BeWo cells grown in 2D to BeWo cells grown in GrowDex®, syncytin-2 was upregulated almost 10-fold in 3D BeWo cells grown for 7 days, a result similar to studies in primary trophoblasts that were grown for 96 hours (Vargas et al., 2009). This result indicates a spontaneous syncytialisation of BeWo cells grown in a nanofibrillar cellulose hydrogel, a novel

discovery in the literature up to this point. Besides an upregulation of Syncytin-2, BeWo cells grown for 7 days in GrowDex® also showed a more than 10-fold upregulation of ERα and a more than 2-fold upregulation of GPR30. Furthermore, hormone expression of β-hCG and E2 in BeWo cells grown in GrowDex® showed a similar expression pattern in a timeframe of 20 days, when compared to a normal human pregnancy of 40 weeks, with β-hCG levels peaking at 7 days in 3D cultures, and falling under E2 levels at 12 days. This pattern is proportional to the pattern in normal human gestation, with peaks of β-hCG at around 12 weeks, or at the end of the 1st trimester, and levels falling to under E2 levels at around 24 weeks, or at the end of the 2nd trimester. These findings indicate that BeWo cells grown in GrowDex® could mimic physiological traits of the gestation period of the human placenta on a smaller scale, therefore providing a valuable model for in *vitro* human placental research.

Although research in the field of placental 3D culture is still lacking, there have been some studies that have analysed growth patterns and different aspects of placental cells and cell lines in 3D using different methods and platforms. HTR8/SVneo cell lines have been grown in Matrigel™ to study trophoblast recruitment to the endometrium (Multhaup et al., 2018), and scaffold-free hanging drop co-culture systems using placental fibroblasts and trophoblasts have shown upregulation of β-hCG secretion compared to 2D models, similar to our data (Muoth et al., 2016). Furthermore, spheroids have been used in order to mimic a 3D environment, using Jar cell lines to show that β-hCG expression increases with this system (H. Wang et al., 2012) and a rotating cell culture system using the placental cell line SGHPL-4 has been developed (Zwezdaryk, Warner, Machado, Morris, & Höner zu Bentrup, 2012). To date, however, BeWo cells have not been grown in a hydrogel and analysed as to their differentiation capacity and structure.

In order to attempt another long-term viable model for placental testing which involves placental tissue, we used term human placental explants embedded in Growdex® in order to extend viability and kept them in culture for over 2 weeks. In order to test for viability, we imaged tissue at 9 and 16 days as well as measuring β-hCG as an indicator for normal function and viability. Structurally, tissues showed intact brush-

border membranes, an essential criterion indicative of viability, as well as keeping levels of β-hCG, indicating viability much past the common limit of 72 hours.

In conclusion, our findings indicate an important role of the 3D environment on BeWo cells. 3D cell culture of the BeWo cell line has promoted spontaneous syncytialisation, suggesting a more physiological milieu for cells to unfold their natural tendencies. Furthermore, we have shown that BeWo cells grown in 3D over 21 days mimic in *vivo* placental hormone expression, with each week representing one gestational trimester, giving promise to the development of a model which encompasses human placental physiology throughout the entire pregnancy. These findings might be similar in explant studies. Given that we have developed a method for prolonging placental explant life in a hydrogel scaffold, we have initiated research into two future methods that could provide the field of placentology with platfoms that are more physiologically similar to human in *vivo* placenta.

Chapter 7

7.1 General discussion

EDCs and their effects on human health is an area of research that is in need of more vigorous comprehensive investigation. In 2012, the World Health Organisation (WHO) and United Nations Environment Programme (UNEP) compiled an assessment on the state of the science on EDCs and mentioned three strands of evidence that caused reasons for concern. Firstly, the fact that there is a high incidence and an increasing trend of disorders related to the endocrine system in humans. Secondly, that there are observable endocrine-related effects in wildlife, and thirdly, that chemicals with endocrine properties have been identified as being linked to diseases in laboratory studies (Bergman et al., 2012). Two of the mentioned diseases linked to EDCs mentioned in this protocol, 2 diabetes and obesity, were increased after EDC exposure, and were diseases that our study have identified as being linked to BPA exposure in placental cells. As *in utero* fetal development is sensitive to chemical exposure and can even determine health outcomes such as diabetes and obesity in the long-term and adult life (Barker, 1997), one of the important messages outlined in the WHO protocol is the importance of windows of exposure. Especially during fetal development, as metabolic rate is increased, and without defense mechanisms that are developed in later life (Newbold, Padilla-Banks, & Jefferson, 2009), exposure to chemicals that can alter the endocrine system can be dangerous. BPA is one of the chemicals that has been linked to a multitude of diseases in children and adults whose pathoaetiology stems from *in utero* BPA exposure. Studies have found links with *in utero* BPA exposure and intrauterine testicular development in humans (Eladak et al., 2018) low sperm concentration and motility in 20-22 year old males (Hart et al., 2018), chronic inflammation in rabbit offspring (Reddivari et al., 2017), alterations in ovarian follicle numbers in mice (Mahalingam et al., 2017), mammary cancer in humans (Paulose, Speroni, Sonnenschein, & Soto, 2015) and changes in the fetal heart transcriptome in rhesus monkeys (Chapalamadugu, VandeVoort, Settles, Robison, & Murdoch, 2014).

BPA is a chemical that is widely used in a vast variety of chemical and industrial processes, which has been shown to affect the human body in a multitude of ways, due to its endocrine disrupting hormonal properties. Due to its ubiquitous nature, it is a compound that warrants intensive and effective research, as it is crucial to limit human exposure as much as possible. In fact, the European Food Safety Authority (EFSA) has identified 12 hazard assessment questions in 2017 regarding the adverse effects of BPA including the mode of action of BPA when considering doses under 100nM, it's toxicokinetic profile in humans, and whether BPA exposure causes any reproductive or developmental outcomes in mammalian animals (Gundert-Remy et al., 2017). With questions remaining about the reproductive and developmental effects of low doses of BPA, studies investigating these effects in tissues that are directly involved in the regulation of human development, such as the placenta, are especially important.

The placenta plays a crucial role in the maintenance of pregnancy, being the interface between mother and fetus, and the organ which is responsible for nutrient and waste exchange. As such, it is vital that placental function is kept throughout pregnancy, as placental pathology is associated with pregnancy complications such as preeclampsia (Vinatier & Monnier, 1995), IUGR (Ganer Herman et al., 2018; Khong & Yee, 1989), and gestational diabetes (Hill, 2018; Ngala, Fondjo, Gmagna, Ghartey, & Awe, 2017), among others. Although all of these pathologies have been researched extensively, they are complex in their aetiology and final conclusions about causes have not been reached. This study has begun the process of the development of new human placental models, a field that will require continuing attention in the future.

Human models of the placenta are lacking when compared to other tissues and organs, and most models of the placenta involve 2D cultures of placental cell lines, short-term placental explant cultures, or animal models. Recent developments in the advance of physiologically relevant tissue and organ models have brought forth models such as organ-on-a-chip, where human organs are represented *in vitro* on a microfluidic chip, using heart (Agarwal, Goss, Cho, McCain, & Parker, 2013), lung (Huh et al., 2010), liver (Domansky et al., 2010), kidney (Jang et al., 2013), brain (Booth & Kim, 2012), gut (Esch et al., 2012) and skin (Ataç et al., 2013) among other organs, as well as organ systems and cancer cells. These methods often employ cells

grown in 3D culture, however the development of these organ-representative models has been absent in placental research. In our study, we have grown BeWo placental cells in two different 3D platforms in order to assess physiological changes associated with 3D growth for future EDC testing, to elucidate how different EDCs as well as mixtures of these can affect different cell types, potentially shedding a light on organ development *in utero* under the influence of environmental toxicants. These platforms have the potential to alter the face of clinical trials, by getting closer than ever before to actual in *vitro* organ development and physiology.

In order to elucidate properties of placental cell lines that could be affected by BPA, as well as similarities to actual placental tissue, we measured base line gene expression and cellular distribution of estrogen receptors in BeWo and JEG-3 cell lines. As previous investigations have not been clear on the presence of estrogen receptors in BeWo and JEG-3 placental cells, we have added valuable knowledge to the research by demonstrating the presence of all three estrogen receptors (ERα, ERβ, GPR30) in both BeWo and JEG-3 cell lines, a prerequisite for the use of these cell lines to test the effects of BPA on the placenta. We have shown that gene expression of ERα is dominant in both BeWo and JEG-3 cell lines, with GPR30 being the next most abundant followed by ERβ. As ERα has been reported to be the primary estrogen receptor in the human placenta (Bukovsky et al., 2003; Schiessl et al., 2006; Yin et al., 2013), we believe that both cell lines accurately represent placenta when it comes to estrogen receptor expression. Furthermore, we showed that all receptors were upregulated after syncytialisation, which likely reflects the known upregulation of estrogen receptors closer to term (Kim et al., 2016), and therefore further validates the BeWo cell line. Furthermore, as we have successfully managed to syncytialise the BeWo cell line, we were able to create a model representing both the cytotrophoblast and syncytiotrophoblast of the placenta, with representative features such as an upregulation of estrogen receptors in differentiated placental cells.

In order to use these cell lines as models to test the effect of BPA on the placenta, we employed the BeWo cell line both when syncytialised and non-syncytialised. The syncytialised model of the placenta more accurately represents the mature placenta, while the non-syncytialised model can be used to model the placenta in earlier stages,

due to an increase in differentiated syncytiotrophoblast later on in pregnancy (Wang & Zhao, 2010). Because human BPA levels in blood, saliva, urine, amniotic fluid and placenta are at nanomolar levels (Edlow, Chen, Smith, Lu, & McElrath, 2012; Schönfelder et al., 2002; Vandenberg et al., 2010), we used 3 nM and 10 nM concentrations of BPA treatment in BeWo and JEG-3 cell lines. We investigated the effects of low levels of BPA in terms of the regulation of estrogen receptor gene expression and mechanisms of action of BPA regarding receptor binding and intracellular signalling. As a xenoestrogen, BPA binds to estrogen receptors. We have found that 24 hour BPA treatment of BeWo cells leads to the significant upregulation of ERα in this cell line, a novel discovery. Studies have shown that E2 can up- or downregulate ERα, depending on the type of tissue (Castles et al., 1997), but regulation of ERα in the placenta as well as placental cell lines is not well researched. Interestingly, our studies have found that ERα is only significantly upregulated when treated with 3 nM BPA and not when treated with 10 nM BPA. These findings are in line with previous studies describing the biphasic effect of BPA, in which lower levels of BPA have a different and non-additive effect when compared to higher levels (Chen et al., 2017; Jeong et al., 2017; Takai et al., 2000; Wang et al., 2015). Some studies have also found an increased effect at lower levels of BPA treatment when compared to higher levels, similar to the effect we have fund in BeWo cells (Hui et al., 2018; Koike, Yanagisawa, Win-Shwe, & Takano, 2018). As ERα upregulation is seen at lower level BPA treatment in BeWo cells, lower levels could actually cause a stronger effect, as there are more receptors for BPA and estrogens to bind to. Although the effect of ER upregulation in the placenta is not widely known, an increase in trophoblastic differentiation to syncytiotrophoblast and functional maturation of the syncytiotrophoblast via estrogen has been reported, with a subsequent increase in hormones such as placental lactogen, which plays a role in mammary development and fetal growth during pregnancy (Musicki, Pepe, & Albrecht, 2003).

In accordance with the finding that ERα was upregulated after 3 nM treatment with BPA, we found that cell proliferation was also significantly increased after treatment with 3 nM BPA, however not with 10 nM BPA treatment. Taken together with the fact that this effect was negated after treatment with the estrogen receptor antagonists G15 and ICI, it is likely that the proliferative effect of BPA is mediated by either ERα or ERβ, and with ERα being the prominent receptor in BeWo cells, and/or via GPR30.

Studies have shown that low levels of GPR30 in placental cells can impair proliferation and invasion, playing a role in the development of PE (Feng et al., 2017; Li et al., 2016; Tong et al., 2016; Zhou et al., 2017) Traditionally, studies have focused on genomic action of estrogen, as receptors were believed to be purely nuclear. More recently, non-genomic action of estrogen has been recorded, whether via the membrane-bound receptor GPR30 or via membrane-bound ERα or ERβ (Vrtačnik, Ostanek, Mencej-Bedrač, & Marc, 2014). As intracellular signaling involving AKT and p38 plays a role in the regulation of cell survival (Coulthard, White, Jones, McDermott, & Burchill, 2009; Cuadrado & Nebreda, 2010; Yu & Cui, 2016), these short-term effects of BPA could play a role in the increased proliferation of BeWo cells treated with BPA. To date there is no literature regarding the effect of low levels of BPA on proliferation and intracellular signaling in BeWo cells, therefore our findings provide a valuable insight into this subject.

Furthermore, our data have shown that proliferation of BeWo cells is a function of AKT activation, as treatment of BeWo cells with an inhibitor of AKT (LY294002) inhibited the proliferative effect of 3 nM BPA. It is well known that P13K/AKT/mTOR signaling, for example, is constitutively activated in different types of cancer, as a pro-survival and proliferative signal, which can be activated by many different types of receptors, including G-protein coupled receptors and receptor tyrosine kinases (LoPiccolo, Blumenthal, Bernstein, & Dennis, 2008) BPA treatment has had differing effects on different types of cells in the literature, however our findings are similar to findings in human thyroid cancer cells, where low levels of BPA (1mM-10 nM) induced proliferation via classical ERs as well as GPR30, and induced proliferation via the activation of AKT, as well as upregulating all three estrogen receptors (Zhang et al., 2017). Investigating how BPA affected levels of phospho-targets in BeWo cells when treated for 15-60 min and found that common targets of estrogen signaling, p-38 and AKT were phosphorylated at different time points, and that there was no increase in ERK1/2 phosphorylation.

Besides the effects of BPA on estrogen receptor expression, proliferation and intracellular signaling, our study investigated changes in gene expression after treatment with BPA using a non-biased screen in the form of microarray. After treatment of non-syncytialised and syncytialised BeWo cells with 3 nM and 10 nM

BPA, differential gene expression was analysed by gene and pathway enrichment, as well as validating upregulated genes of interest. Interestingly, differential gene expression was most prominent in non-syncytialised cells treated with 3 nM BPA, indicating that lower levels of BPA and treatment of undifferentiated cells were favourable conditions for changes in gene expression. RhoA and Rac1 signaling pathways, insulin signaling, insulin resistance, leptin signaling and differentiation of white and brown adipose tissue pathways were the most functionally enriched entries. RhoA and Rac1 signaling pathways are involved in the regulation of a multitude of functions within the cell, including endosomal trafficking, actin polymerization, cell survival and cell cycle progression (Schwartz, 2004). In terms of proliferation, RhoA promotes proliferation by inhibiting p21 expression and inducing cyclin D1 expression thereby promoting G1 entry and progression into S-phase (Etienne-Manneville & Hall, 2002; M F Olson et al., 1995) and Rac has the potential to stimulate proliferation of cells via the activation of JNK/SAPK (Olson et al., 1995).

Interestingly, the pathways enriched after 3 nM BPA treatment have been shown to be linked in a multitude of studies. Leptin and insulin work together in controlling glucose metabolism, thereby regulating fetal growth and development, with umbilical cord leptin levels correlating positively with neonate fat mass and body weight (Guzmán-Bárcenas et al., 2016) and increased maternal glucose levels contributing to large for gestational age pregnancies (Migda, Migda, Migda, & Wender-Ozegowska, 2017).

RhoA signaling also has been shown to be involved in regulation of insulin, with the RhoA/ROCK pathway mediating skeletal muscle insulin resistance (Chun et al., 2011; Tao et al., 2015) and mediating insulin release in mouse pancreatic β-cells (Xiaofang Liu et al., 2014). RhoA and its effector ROCK1 have also been linked to leptin, with leptin being one of the activators of RhoA and thereby inducing processes such as cell invasion, reorganization of the cytoskeleton, homeostasis of feeding behaviour and increasing permeability of colon tight junctions (Ghasemi, Isaac Hashemy, Aghaei, & Panjehpour, 2017; Huang et al., 2012; Le Dréan et al., 2014). RhoA is also involved in regulation of leptin function when it comes to the promotion by leptin of hypertension, atherosclerosis and reactive oxygen species (ROS) generation (Ghantous et al., 2015). Rac1 similarly has been found to be involved in regulation of insulin and insulin

resistance development, with Rac1 being activated by insulin and increasing GLUT4 cellular traffic and Rac activation being impaired in insulin resistance (Chiu et al., 2011; JeBailey et al., 2007; Ueda et al., 2010, 2008), as well as being activated by insulin in mouse adipose tissue (Takenaka et al., 2017). It plays a role in excess ROS accumulation in diabetic conditions (Sidarala & Kowluru, 2017), therefore being implicated in states of chronic inflammation, making it a player in metabolic syndrome which includes type 2 diabetes (Zhou et al., 2015).

Taken together, these findings highlight the capacity of BPA to affect the BeWo cell genome. These changes imply a role of BPA in influencing the metabolism as well as proliferation of placental cells, factors that could immensely affect a fetus during pregnancy as well as determine the outcome of the pregnancy itself. Insulin signaling is a pathway which has been demonstrated to play a major role during pregnancy, as gestational diabetes mellitus, for example, is a disease which can have severe effects on the fetus, pregnancy, and long-term effects on both mother and child, including fetal macrosomia, maternal preeclampsia, neonatal hyperglycemia and respiratory distress syndrome, the development of type 2 diabetes of the mother after pregnancy, as well as an increased risk of the development of obesity and abnormal glucose metabolism of the offspring whether in childhood, adolescence or adulthood (Gilmartin, Ural, & Repke, 2008; Petry, 2018; Schmidt et al., 2001) . As obesity is one of the risk factors of pregnant women for developing gestational diabetes, high leptin levels may also be a contributing factor when it comes to the aetiology of gestational diabetes. In fact, recent studies have shown the relationship between higher placental leptin and the development of insulin resistance and gestational diabetes, as well as showing that levels of leptin in the placenta were higher in macrosomic offspring than in normal weight offspring (Shang, Dong, & Hou, 2018; Tsiotra et al., 2018).

Lastly, we have attempted to develop a relevant model of the human placenta for future EDC testing. By growing BeWo cells on a 3D scaffold using Growdex®, we enabled cells to develop a more physiological 3D form with increased cell-to-cell-interactions, forming cell clusters that syncytialise to become differentiated. These cells grown in 3D mimicked the human placenta when it came to hormonal secretion of β-hCG and E2, with every week of growth representing a trimester during

pregnancy, therefore indicating that this platform is useful in simulating the human placenta in vivo. Furthermore, we have managed to keep human placental explants viable in culture for 16 days providing another physiological platform for long-term testing. These models have initiated research into providing new and physiologically relevant platforms that can mimic human placenta, which are needed in order to address questions such as effects of chemical exposure during pregnancy, and must be practical, efficient and, most importantly show similar traits to placental tissue in *vitro*. Our models show promise in terms of hormone secretion patterns as well as structural fidelity but will need to be further developed.

7.2 Study limitations

We acknowledge that this study has some limitations, which could be addressed in future studies. Firstly, although it is valuable to study the effects of a single chemical on the human placenta, the reality of the *in vivo* situation is more complex. Humans are exposed to a mixture of EDCs on a daily basis (Ribeiro, Ladeira, & Viegas, 2017; Webster, 2013), and these combinations could have an additive effect or other more complex interaction. Secondly, working with the choriocarcinoma cell line BeWo always warrants caution when interpreting results, as even though traits are very similar to placental cells, the cell line is derived from choriocarcinoma, and therefore differs in its genome and karyotype. Furthermore, we did not investigate the effects of BPA long-term, as in all our experiments we assessed effects of BPA after 24 hours of exposure. As *in vivo* exposure to BPA is more chronic than acute, it would be interesting and valuable to assess effects after a series of days or even weeks. As it is often not possible to use 2D cell lines to investigate long-term effects of a substance, other models would have to be used as a more robust model for this endeavour. When investigating effects of BPA on placental tissue, only one placenta was analysed. Due to patient variability, a vast amount of placentae would need to be used in order to discern a pattern. Lastly, some of our experiments of gene regulation were not followed by studies investigating protein regulation, which would give a more complete picture of the effects of BPA on the proteome.

7.3 Future studies

This study evaluates the effects of BPA on the placenta, however there is a large scope of possible effects to evaluate. Therefore, this study can be seen as having made first steps into how BPA affects BeWo cells, as well as addressing the need for more physiologically relevant models and laying the foundation to investigate much needed 3D platforms. Therefore, there is room and need for future studies to build on data that has emerged from our study.

Firstly, our study did not address some properties of cells which can be affected by hormones and chemicals, such as migration and differentiation. Future studies need to investigate how BPA can affect the migration of the BeWo cell line, as well as whether BPA plays a role in the differentiation of the trophoblast. Other properties such as epigenetic DNA changes could play a role in the effects of BPA, and warrant investigating. Furthermore, more recent developments in the function of BPA, such as the discovery that BPA can bind to estrogen-related receptor γ (ERRγ) (Tohmé et al., 2014) will play a role in determining the molecular function of BPA in the future. Therefore, there is a vast variety of studies that should still be done in BeWo cells to create a more complete picture of the effects of BPA on this cell line.

Future studies should also employ models that can mimic the placental environment more accurately, such as the regulation of flow, as well as the co-culture of cells within the same environment, as signaling between cells can vastly influence the physiology of an organ. Specifically, organ-on-a-chip technologies are being employed more frequently in recent studies, and placenta-on-a-chip systems are slowly emerging, for example by using JEG-3 cells and human umbilical vein (HUVEC) cells to recreate the placental barrier (Lee et al., 2016). Not only are organ-on-a-chip models being used for research, but organ systems such as intestine-liver-kidney-blood-brain-barrier-skeletal-muscle systems (Vernetti et al., 2017) are emerging at a rapid pace. Future studies coupling placenta with fetal tissue or even more complex systems are needed in order to develop a more complete picture of chemical effects during human pregnancy. More simple models such as co-cultures of placental and fetal cells grown in 3D would also give valuable insights. Ideally, future techniques would employ a combination of chip technology and 3D culture, being able to regulate as many

physiological variables as possible. These developments would not only improve experimental readings in terms of accuracy, but drive the replacement of animal studies in the field of placenta research, which often uses mammals and higher primates such as baboons, a serious ethical concern.

Future studies should also take into account the different types of cells used when setting up models. Although cell lines are useful, primary cells not only are taken from human placenta directly and have not gone through genomic changes to make them immortalised but are variable from patient to patient and can therefore represent the human placenta more broadly, when large numbers of samples are obtained. Therefore, if possible, a move away from cell lines and towards primary cells grown in a 3D environment would be useful. Explants also need to be sampled from a high number of patients, in order to discern effects across all samples, and understand common effects of environmental toxicants. Furthermore, both primary cells and explants should be taken from patients with pre-existing conditions in pregnancy or outside of pregnancy, to understand differences in patient populations with disease.

Finally, mixtures of chemical substances that women are exposed to during pregnancy will need to be investigated, as their potential harm and ability to exert additive effects are still not well known to date. Studies like these are crucial, as the only way to fully understand how chemicals can affect human pregnancies is to investigate their effects in the context of human exposure to a multitude of different environmental chemicals as well as endogenous processes. As exposures during pregnancy as well as pregnancies themselves are heterogeneous due to a multitude of variables, the ultimate goal will be to create models that are population and patient specific, in order to work towards a more individualised approach to placental testing.

References

Acconcia, F., & Marino, M. (2011). The Effects of 17β-estradiol in Cancer are Mediated by Estrogen Receptor Signaling at the Plasma Membrane. *Frontiers in Physiology*, *2*, 30. https://doi.org/10.3389/fphys.2011.00030

Acerini, C. L., & Hughes, I. A. (2006). Endocrine disrupting chemicals: a new and emerging public health problem? *Archives of Disease in Childhood*, *91*(8), 633–41. https://doi.org/10.1136/adc.2005.088500

Adlanmerini, M., Solinhac, R., Abot, A., Fabre, A., Raymond-Letron, I., Guihot, A.-L., … Lenfant, F. (2014). Mutation of the palmitoylation site of estrogen receptor α in vivo reveals tissue-specific roles for membrane versus nuclear actions. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(2), E283-90. https://doi.org/10.1073/pnas.1322057111

Agarwal, A., Goss, J. A., Cho, A., McCain, M. L., & Parker, K. K. (2013). Microfluidic heart on a chip for higher throughput pharmacological studies. *Lab on a Chip*, *13*(18), 3599–608. https://doi.org/10.1039/c3lc50350j

Airaksinen, R., Rantakokko, P., Eriksson, J. G., Blomstedt, P., Kajantie, E., & Kiviranta, H. (2011). Association between type 2 diabetes and exposure to persistent organic pollutants. *Diabetes Care*, *34*(9), 1972–9. https://doi.org/10.2337/dc10-2303

Al-Nasiry, S., Spitz, B., Hanssens, M., Luyten, C., & Pijnenborg, R. (2006). Differential effects of inducers of syncytialization and apoptosis on BeWo and JEG-3 choriocarcinoma cells. *Human Reproduction*, *21*(1), 193–201. https://doi.org/10.1093/humrep/dei272

Al-Nasiry, S., Vercruysse, L., Hanssens, M., Luyten, C., & Pijnenborg, R. (2009). Interstitial Trophoblastic Cell Fusion and E-cadherin Immunostaining in the Placental Bed of Normal and Hypertensive Pregnancies. *Placenta*, *30*(8), 719–725. https://doi.org/10.1016/j.placenta.2009.05.006

Alahari, S. K., Reddig, P. J., & Juliano, R. L. (2002). Biological aspects of signal transduction by cell adhesion receptors. *International Review of Cytology*, *220*, 145–184. https://doi.org/10.1016/S0074-7696(02)20005-4

Alonso-Magdalena, P., Laribi, O., Ropero, A. B., Fuentes, E., Ripoll, C., Soria, B., & Nadal, A. (2005). Low doses of bisphenol A and diethylstilbestrol impair Ca2+ signals in pancreatic alpha-cells through a nonclassical membrane estrogen receptor within intact islets of Langerhans. *Environmental Health Perspectives*, *113*(8), 969–77. https://doi.org/10.1289/EHP.8002

Alonso-Magdalena, P., Quesada, I., & Nadal, Á. (2015). Prenatal Exposure to BPA and Offspring Outcomes: The Diabesogenic Behavior of BPA. *Dose-Response : A Publication of International Hormesis Society*, *13*(2), 1559325815590395. https://doi.org/10.1177/1559325815590395

Alonso-Magdalena, P., Ropero, A. B., Soriano, S., García-Arévalo, M., Ripoll, C., Fuentes, E., … Nadal, Á. (2012). Bisphenol-A acts as a potent estrogen via non-classical estrogen triggered pathways. *Molecular and Cellular Endocrinology*, *355*(2), 201–207. https://doi.org/10.1016/j.mce.2011.12.012

Aneck-Hahn, N. H., Schulenburg, G. W., Bornman, M. S., Farias, P., & De Jager, C. (2006). Impaired Semen Quality Associated With Environmental DDT Exposure in Young Men Living in a Malaria Area in the Limpopo Province, South Africa. *Journal of Andrology*, *28*(3), 423–434. https://doi.org/10.2164/jandrol.106.001701

Aplin, J. D., Haigh, T., Vicovac, L., Church, H. J., & Jones, C. J. P. (1998). Anchorage in the developing placenta: An overlooked determinant of pregnancy outcome? *Human Fertility*, *1*(1), 75–79. https://doi.org/10.1080/1464727982000198161

Arrebola, J. P., Cuellar, M., Bonde, J. P., González-Alzaga, B., & Mercado, L. A. (2016). Associations of maternal o , p ′-DDT and p , p ′-DDE levels with birth outcomes in a Bolivian cohort. *Environmental Research*, *151*, 469–477. https://doi.org/10.1016/j.envres.2016.08.008

Ataç, B., Wagner, I., Horland, R., Lauster, R., Marx, U., Tonevitsky, A. G., … Lindner, G. (2013). Skin and hair on-a-chip: in vitro skin models versus ex vivo tissue maintenance with dynamic perfusion. *Lab on a Chip*, *13*(18), 3555–61. https://doi.org/10.1039/c3lc50227a

Bai, P. Y., Wittert, G., Taylor, A. W., Martin, S. A., Milne, R. W., Jenkins, A. J., … Shi, Z. (2017). The association between total phthalate concentration and non-communicable diseases and chronic inflammation in South Australian urban dwelling men. *Environmental Research*, *158*, 366–372. https://doi.org/10.1016/j.envres.2017.06.021

Balakrishnan, B., Henare, K., Thorstensen, E. B., Ponnampalam, A. P., & Mitchell, M. D. (2010). Transfer of bisphenol A across the human placenta. *American Journal of Obstetrics and Gynecology*, *202*(4), 393.e1-393.e7. https://doi.org/10.1016/j.ajog.2010.01.025

Barker, D. (1992). Fetal growth and adult disease. *BJOG: An International Journal of Obstetrics and Gynaecology*, *99*(4), 275–276. https://doi.org/10.1111/j.1471-0528.1992.tb13719.x

Barker, D. J. P. (1997). Maternal nutrition, fetal nutrition, and disease in later life. *Nutrition*, *13*(9), 807–813. https://doi.org/10.1016/S0899-9007(97)00193-7

Barton, M. (2016). Not lost in translation: Emerging clinical importance of the G protein-coupled estrogen receptor GPER. *Steroids*, *111*, 37–45. https://doi.org/10.1016/j.steroids.2016.02.016

Belcher, S. M., Burton, C. C., Cookman, C. J., Kirby, M., Miranda, G. L., Saeed, F. O., & Wray, K. E. (2017). Estrogen and soy isoflavonoids decrease sensitivity of medulloblastoma and central nervous system primitive neuroectodermal tumor cells to chemotherapeutic cytotoxicity. *BMC Pharmacology & Toxicology*, *18*(1), 63. https://doi.org/10.1186/s40360-017-0160-7

Bellavia, A., Hauser, R., Seely, E. W., Meeker, J. D., Ferguson, K. K., Mcelrath, T. F.,

& James-Todd, T. (2017). Urinary phthalate metabolite concentrations and maternal weight during early pregnancy. https://doi.org/10.1016/j.ijheh.2017.09.005

Ben-Jonathan, N., & Steinmetz, R. (1998). Xenoestrogens: The emerging story of bisphenol A. *Trends in Endocrinology and Metabolism*, *9*(3), 124–128. https://doi.org/10.1016/S1043-2760(98)00029-0

Benachour, N., & Aris, A. (2009). Toxic effects of low doses of Bisphenol-A on human placental cells. *Toxicology and Applied Pharmacology*, *241*(3), 322–328. https://doi.org/10.1016/j.taap.2009.09.005

Benjamin, S., Masai, E., Kamimura, N., Takahashi, K., Anderson, R. C., & Faisal, P. A. (2017). Phthalates impact human health: Epidemiological evidences and plausible mechanism of action. *Journal of Hazardous Materials*, *340*(340), 360–383. https://doi.org/10.1016/j.jhazmat.2017.06.036

Berger, K. P., Kogut, K. R., Bradman, A., She, J., Gavin, Q., Zahedi, R., … Harley, K. G. (2018). Personal care product use as a predictor of urinary concentrations of certain phthalates, parabens, and phenols in the HERMOSA study. *Journal of Exposure Science & Environmental Epidemiology*, 1. https://doi.org/10.1038/s41370-017-0003-z

Berger, R. G., Foster, W. G., & deCatanzaro, D. (2010). Bisphenol-A exposure during the period of blastocyst implantation alters uterine morphology and perturbs measures of estrogen and progesterone receptor expression in mice. *Reproductive Toxicology*, *30*(3), 393–400. https://doi.org/10.1016/j.reprotox.2010.06.006

Berger, R. G., Hancock, T., & deCatanzaro, D. (2007). Influence of oral and subcutaneous bisphenol-A on intrauterine implantation of fertilized ova in inseminated female mice. *Reproductive Toxicology*, *23*(2), 138–144. https://doi.org/10.1016/j.reprotox.2006.09.005

Berger, R. G., Shaw, J., & deCatanzaro, D. (2008). Impact of acute bisphenol-A exposure upon intrauterine implantation of fertilized ova and urinary levels of progesterone and 17β-estradiol. *Reproductive Toxicology*, *26*(2), 94–99. https://doi.org/10.1016/j.reprotox.2008.06.007

Berger, V. K., Sparks, T. N., Jelin, A. C., Derderian, C., Jeanty, C., Gosnell, K., … Gonzalez, J. M. (2018). Non-Immune Hydrops Fetalis: Do Placentomegaly and Polyhydramnios Matter? *Journal of Ultrasound in Medicine*, *37*(5), 1185–1191. https://doi.org/10.1002/jum.14462

Bergman, Å., Heindel, J. J., Jobling, S., Kidd, K. A., & Zoeller, R. T. (2012). State of the Science of Endocrine Disrupting Chemicals 2012 Summary for Decision-Makers inter-organization programme for the sound management of chemicals. Retrieved from https://wedocs.unep.org/bitstream/handle/20.500.11822/17015/State_Science_Endocrine_Disrupting_Chemicals.pdf?sequence=1&isAllowed=y

Bergman, Å., Heindel, J. J., Jobling, S., Kidd, K. A., Zoeller, R. T., World Health Organization., & United Nations Environment Programme. (2013). *State of the*

science of endocrine disrupting chemicals - 2012 : an assessment of the state of the science of endocrine disruptors prepared by a group of experts for the United Nations Environment Programme (UNEP) and WHO. United National Environment Programme.

Betancourt, A. M., Mobley, J. A., Russo, J., & Lamartiniere, C. A. (2010). Proteomic analysis in mammary glands of rat offspring exposed in utero to bisphenol A. *Journal of Proteomics*, *73*(6), 1241–1253. https://doi.org/10.1016/j.jprot.2010.02.020

Bhattacharya, M., Malinen, M. M., Lauren, P., Lou, Y.-R., Kuisma, S. W., Kanninen, L., … Yliperttula, M. (2012). Nanofibrillar cellulose hydrogel promotes three-dimensional liver cell culture. *Journal of Controlled Release*, *164*(3), 291–298. https://doi.org/10.1016/j.jconrel.2012.06.039

Bigsby, R., Chapin, R. E., Daston, G. P., Davis, B. J., Gorski, J., Gray, L. E., … vom Saal, F. S. (1999). Evaluating the effects of endocrine disruptors on endocrine function during development. *Environmental Health Perspectives*, *107 Suppl*(Suppl 4), 613–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10421771

Björnström, L., & Sjöberg, M. (2005). Mechanisms of Estrogen Receptor Signaling: Convergence of Genomic and Nongenomic Actions on Target Genes. *Molecular Endocrinology*, *19*(4), 833–842. https://doi.org/10.1210/me.2004-0486

Booth, R., & Kim, H. (2012). Characterization of a microfluidic in vitro model of the blood-brain barrier (μBBB). *Lab on a Chip*, *12*(10), 1784–92. https://doi.org/10.1039/c2lc40094d

Brett, K. E., Ferraro, Z. M., Yockell-Lelievre, J., Gruslin, A., & Adamo, K. B. (2014). Maternal-fetal nutrient transport in pregnancy pathologies: the role of the placenta. *International Journal of Molecular Sciences*, *15*(9), 16153–85. https://doi.org/10.3390/ijms150916153

Brew, O., & Sullivan, M. H. F. (2017). Oxygen and tissue culture affect placental gene expression. *Placenta*, *55*, 13–20. https://doi.org/10.1016/j.placenta.2017.04.024

Brieño-Enríquez, M. A., Robles, P., Camats-Tarruella, N., García-Cruz, R., Roig, I., Cabero, L., … Caldés, M. G. (2011). Human meiotic progression and recombination are affected by Bisphenol A exposure during in vitro human oocyte development. *Human Reproduction*, *26*(10), 2807–2818. https://doi.org/10.1093/humrep/der249

Brown, L. M., Lacey, H. A., Baker, P. N., & Crocker, I. P. (2005). E-cadherin in the assessment of aberrant placental cytotrophoblast turnover in pregnancies complicated by pre-eclampsia. *Histochemistry and Cell Biology*, *124*(6), 499–506. https://doi.org/10.1007/s00418-005-0051-7

Brucker-Davis, F., Wagner-Mahler, K., Delattre, I., Ducot, B., Ferrari, P., Bongain, A., … Fenichel, P. (2008). Cryptorchidism at birth in Nice area (France) is associated with higher prenatal exposure to PCBs and DDE, as assessed by colostrum concentrations. *Human Reproduction*, *23*(8), 1708–1718. https://doi.org/10.1093/humrep/den186

Bukovsky, A., Cekanova, M., Caudle, M. R., Wimalasena, J., Foster, J. S., Henley, D. C., & Elder, R. F. (2003). Expression and localization of estrogen receptor-alpha protein in normal and abnormal term placentae and stimulation of trophoblast differentiation by estradiol. *Reproductive Biology and Endocrinology*, *1*, 1–18. https://doi.org/10.1186/1477-7827-1-13

Bullens, D. M., Truyen, E., Coteur, L., Dilissen, E., Hellings, P. W., Dupont, L. J., & Ceuppens, J. L. (2006). IL-17 mRNA in sputum of asthmatic patients: linking T cell driven inflammation and granulocytic influx? *Respiratory Research*, *7*(1), 135. https://doi.org/10.1186/1465-9921-7-135

Burton, G. J., & Fowden, A. L. (2015). The placenta: a multifaceted, transient organ. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *370*(1663), 20140066. https://doi.org/10.1098/rstb.2014.0066

Candelier, J.-J., Frappart, L., Diatta, A. L., Yadaden, T., Cissé, M.-L., Afoutou, J.-M., … Coullin, P. (n.d.). Differential expression of E-cadherin, β-catenin, and Lewis x between invasive hydatidiform moles and post-molar choriocarcinomas. https://doi.org/10.1007/s00428-013-1427-z

Cantonwine, D. E., Meeker, J. D., Ferguson, K. K., Mukherjee, B., Hauser, R., & McElrath, T. F. (2016). Urinary Concentrations of Bisphenol A and Phthalate Metabolites Measured during Pregnancy and Risk of Preeclampsia. *Environmental Health Perspectives*, *124*(10), 1651–1655. https://doi.org/10.1289/EHP188

Cantonwine, D. E. mmm, Hauser, R., & Meeker, J. D. (2013). Bisphenol A and Human Reproductive Health. *Expert Rev Obstet Gynecol*, *8*(4), 329–335. https://doi.org/10.1586/17474108.2013.811939.Bisphenol

Carter, A. M. (2007). Animal Models of Human Placentation - A Review. *Placenta*, *28*(SUPPL.), S41–S47. https://doi.org/10.1016/j.placenta.2006.11.002

Carter, B. S., Moores, R. R., & Battaglia, F. C. (1991). Placental transport and fetal and placental metabolism of amino acids. *The Journal of Nutritional Biochemistry*, *2*(1), 4–13. https://doi.org/10.1016/0955-2863(91)90041-3

Casals-Casas, C., & Desvergne, B. (2011). Endocrine Disruptors: From Endocrine to Metabolic Disruption. *Annual Review of Physiology*, *73*(1), 135–162. https://doi.org/10.1146/annurev-physiol-012110-142200

Castles, C. G., Oesterreich, S., Hansen, R., & Fuqua, S. a W. (1997). Auto-regulation of the estrogen receptor promoter. *The Journal of Steroid Biochemistry and Molecular Biology*, *62*(2–3), 155–63. https://doi.org/10.1016/S0960-0760(97)00023-X

Chabaud, M., Durand, J. M., Buchs, N., Fossiez, F., Page, G., Frappart, L., & Miossec, P. (1999). Human interleukin-17: A T cell-derived proinflammatory cytokine produced by the rheumatoid synovium. *Arthritis & Rheumatism*, *42*(5), 963–970. https://doi.org/10.1002/1529-0131(199905)42:5<963::AID-ANR15>3.0.CO;2-E

Chambliss, K. L., Yuhanna, I. S., Mineo, C., Liu, P., German, Z., Sherman, T. S., … Shaul, P. W. (2000). Estrogen receptor alpha and endothelial nitric oxide synthase

are organized into a functional signaling module in caveolae. *Circulation Research*, *87*(11), E44-52. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11090554

Chang, W.-H., Wu, M.-H., Pan, H.-A., Guo, P.-L., & Lee, C.-C. (2017). Semen quality and insulin-like factor 3: Associations with urinary and seminal levels of phthalate metabolites in adult males. *Chemosphere*, *173*, 594–602. https://doi.org/10.1016/j.chemosphere.2017.01.056

Chang, W.-L., Liu, Y.-W., Dang, Y.-L., Jiang, X.-X., Xu, H., Huang, X., … Wang, H. (2018). PLAC8, a new marker for human interstitial extravillous trophoblast cells, promotes their invasion and migration. *Development (Cambridge, England)*, *145*(2). https://doi.org/10.1242/dev.148932

Chang, W.-L., Yang, Q., Zhang, H., Lin, H.-Y., Zhou, Z., Lu, X., … Wang, H. (2014). Role of placenta-specific protein 1 in trophoblast invasion and migration. *Reproduction (Cambridge, England)*, *148*(4), 343–52. https://doi.org/10.1530/REP-14-0052

Chapalamadugu, K. C., VandeVoort, C. A., Settles, M. L., Robison, B. D., & Murdoch, G. K. (2014). Maternal Bisphenol A Exposure Impacts the Fetal Heart Transcriptome. *PLoS ONE*, *9*(2), e89096. https://doi.org/10.1371/journal.pone.0089096

Chen, E. Y., Tan, C. M., Kou, Y., Duan, Q., Wang, Z., Meirelles, G., … Ma'ayan, A. (2013). Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC Bioinformatics*, *14*(1), 128. https://doi.org/10.1186/1471-2105-14-128

Chen, X., Wang, Y., Xu, F., Wei, X., Zhang, J., Wang, C., … Wang, Q. (2017). The Rapid Effect of Bisphenol-A on Long-Term Potentiation in Hippocampus Involves Estrogen Receptors and ERK Activation. *Neural Plasticity*, *2017*, 5196958. https://doi.org/10.1155/2017/5196958

Cheong, A., Zhang, X., Cheung, Y.-Y., Tang, W.-Y., Chen, J., Ye, S.-H., … Ho, S.-M. (2016). DNA methylome changes by estradiol benzoate and bisphenol A links early-life environmental exposures to prostate cancer risk. *Epigenetics*, *11*(9), 674–689. https://doi.org/10.1080/15592294.2016.1208891

Chiu, T. T., Jensen, T. E., Sylow, L., Richter, E. A., & Klip, A. (2011). Rac1 signalling towards GLUT4/glucose uptake in skeletal muscle. *Cellular Signalling*, *23*, 1546–1554. https://doi.org/10.1016/j.cellsig.2011.05.022

Cho, G. J., Yoo, S. W., Hong, S. C., Oh, M.-J., Kim, T., Kim, H. J., … Kim, S. H. (2006). Correlations between umbilical and maternal serum resistin levels and neonatal birth weight. *Acta Obstetricia et Gynecologica Scandinavica*, *85*(9), 1051–1056. https://doi.org/10.1080/00016340500470150

Christofori, G., & Semb, H. (1999). The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene. *Trends in Biochemical Sciences*, *24*(2), 73–76. https://doi.org/10.1016/S0968-0004(98)01343-7

Chun, K.-H., Choi, K.-D., Lee, D.-H., Jung, Y., Henry, R. R., Ciaraldi, T. P., & Kim, Y.-

B. (2011). In vivo activation of ROCK1 by insulin is impaired in skeletal muscle of humans with type 2 diabetes. *American Journal of Physiology. Endocrinology and Metabolism*, *300*(3), E536-42. https://doi.org/10.1152/ajpendo.00538.2010

Cimarosti, H., O ', R. D., Ae, S. S., Jones, N. M., Horn, A. P., Fabrício, A., … Salbego, C. (2006). The effects of estradiol on estrogen receptor and glutamate transporter expression in organotypic hippocampal cultures exposed to oxygen-glucose deprivation. *Neurochemical Research*, *31*(4), 483–490. https://doi.org/10.1007/s11064-006-9043-9

Coburn, C. G., Gillard, E. R., & Currás-Collazo, M. C. (2005). Dietary Exposure to Aroclor 1254 Alters Central and Peripheral Vasopressin Release in Response to Dehydration in the Rat. *TOXICOLOGICAL SCIENCES*, *84*, 149–156. https://doi.org/10.1093/toxsci/kfi046

Codru, N., Schymura, M. J., Negoita, S., Akwesasne Task Force on Environment, R., Rej, R., & Carpenter, D. O. (2007). Diabetes in relation to serum levels of polychlorinated biphenyls and chlorinated pesticides in adult Native Americans. *Environmental Health Perspectives*, *115*(10), 1442–7. https://doi.org/10.1289/ehp.10315

Cohn, B. A., La Merrill, M., Krigbaum, N. Y., Yeh, G., Park, J.-S., Zimmermann, L., & Cirillo, P. M. (2015). DDT Exposure in Utero and Breast Cancer. *The Journal of Clinical Endocrinology and Metabolism*, *100*(8), 2865–72. https://doi.org/10.1210/jc.2015-1841

Coker, E., Chevrier, J., Rauch, S., Bradman, A., Obida, M., Crause, M., … Eskenazi, B. (2018). Association between prenatal exposure to multiple insecticides and child body weight and body composition in the VHEMBE South African birth cohort. *Bornman@up.Ac.Za (R. Bornman), Eskenazi@berkeley.Edu (B. Eskenazi). Environment International*, *113*, 122–132. https://doi.org/10.1016/j.envint.2018.01.016

Cole, L. A. (2012). HCG, the wonder of today's science. *Reproductive Biology and Endocrinology*, *10*(1), 24. https://doi.org/10.1186/1477-7827-10-24

Cornelius, D. C., & Lamarca, B. (2014). TH17- and IL-17- mediated autoantibodies and placental oxidative stress play a role in the pathophysiology of pre-eclampsia. *Minerva Ginecologica*, *66*(3), 243–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/24971780

Costa, L. G. (2015). The neurotoxicity of organochlorine and pyrethroid pesticides (pp. 135–148). https://doi.org/10.1016/B978-0-444-62627-1.00009-3

Costa, M. A. (2016). The endocrine function of human placenta: An overview. *Reproductive BioMedicine Online*, *32*(1), 14–43. https://doi.org/10.1016/j.rbmo.2015.10.005

Coulthard, L. R., White, D. E., Jones, D. L., McDermott, M. F., & Burchill, S. A. (2009). p38(MAPK): stress responses from molecular mechanisms to therapeutics. *Trends in Molecular Medicine*, *15*(8), 369–79. https://doi.org/10.1016/j.molmed.2009.06.005

Coutifaris, C., Kao, L. C., Sehdev, H. M., Chin, U., Babalola, G. O., Blaschuk, O. W., & Strauss, J. F. (1991). E-cadherin expression during the differentiation of human trophoblasts. *Development (Cambridge, England)*, *113*(3), 767–77. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1821848

Crews, D., Willingham, E., & Skipper, J. K. (2000). ENDOCRINE DISRUPTORS: PRESENT ISSUES, FUTURE DIRECTIONS.: EBSCOhost. Retrieved January 18, 2018, from http://web.b.ebscohost.com.ezproxy.brunel.ac.uk/ehost/pdfviewer/pdfviewer?vid=1&sid=03f92798-b91b-438d-bdbc-82c6ad0e9915@sessionmgr104

Crinnion, W. J. (2011). Polychlorinated biphenyls: persistent pollutants with immunological, neurological, and endocrinological consequences. *Alternative Medicine Review : A Journal of Clinical Therapeutic*, *16*(1), 5–13. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/21438643

Cromwell, H. C., Johnson, A., McKnight, L., Horinek, M., Asbrock, C., Burt, S., … Meserve, L. A. (2007). Effects of polychlorinated biphenyls on maternal odor conditioning in rat pups. *Physiology & Behavior*, *91*(5), 658–66. https://doi.org/10.1016/j.physbeh.2007.03.029

Cross, J., Werb, Z., & Fisher, S. (1994). Implantation and the placenta: key pieces of the development puzzle. *Science*, *266*(5190), 1508–1518. https://doi.org/10.1126/science.7985020

Cuadrado, A., & Nebreda, A. R. (2010). Mechanisms and functions of p38 MAPK signalling. *Biochemical Journal*, *429*(3), 403–417. https://doi.org/10.1042/BJ20100323

D'Aloisio, A. A., Baird, D. D., DeRoo, L. A., & Sandler, D. P. (2010). Association of intrauterine and early-life exposures with diagnosis of uterine leiomyomata by 35 years of age in the Sister Study. *Environmental Health Perspectives*, *118*(3), 375–81. https://doi.org/10.1289/ehp.0901423

Darmochwal-Kolarz, D., Michalak, M., Kolarz, B., Przegalinska-Kalamucka, M., Bojarska-Junak, A., Sliwa, D., & Oleszczuk, J. (2017). The Role of Interleukin-17, Interleukin-23, and Transforming Growth Factor-β in Pregnancy Complicated by Placental Insufficiency. *BioMed Research International*, *2017*, 6904325. https://doi.org/10.1155/2017/6904325

Das, N., Datta, N., Chatterjee, U., & Ghosh, M. K. (2016). Estrogen receptor alpha transcriptionally activates casein kinase 2 alpha: A pivotal regulator of promyelocytic leukaemia protein (PML) and AKT in oncogenesis. *Cellular Signalling*, *28*(6), 675–687. https://doi.org/10.1016/j.cellsig.2016.03.007

Davies, J. E., Pollheimer, J., Yong, H. E. J., Kokkinos, M. I., Kalionis, B., Knöfler, M., & Murthi, P. (2016). Epithelial-mesenchymal transition during extravillous trophoblast differentiation. *Cell Adhesion & Migration*, *10*(3), 310–21. https://doi.org/10.1080/19336918.2016.1170258

Deb, M., Sengupta, D., Kar, S., Rath, S. K., Parbin, S., Shilpi, A., … Patra, S. K. (2014). Elucidation of caveolin 1 both as a tumor suppressor and metastasis promoter in light of epigenetic modulators. *Tumor Biology*, *35*(12), 12031–12047.

https://doi.org/10.1007/s13277-014-2502-z

DeBenedictis, B., Guan, H., & Yang, K. (2016). Prenatal Exposure to Bisphenol A Disrupts Mouse Fetal Liver Maturation in a Sex-Specific Manner. *Journal of Cellular Biochemistry*, *117*(2), 344–350. https://doi.org/10.1002/jcb.25276

Denham, M., Schell, L. M., Deane, G., Gallo, M. V, Ravenscroft, J., DeCaprio, A. P., & Akwesasne Task Force on the Environment. (2005). Relationship of Lead, Mercury, Mirex, Dichlorodiphenyldichloroethylene, Hexachlorobenzene, and Polychlorinated Biphenyls to Timing of Menarche Among Akwesasne Mohawk Girls. *PEDIATRICS*, *115*(2), e127–e134. https://doi.org/10.1542/peds.2004-1161

Dennis, M. K., Burai, R., Ramesh, C., Petrie, W. K., Alcon, S. N., Nayak, T. K., … Prossnitz, E. R. (2009). In vivo effects of a GPR30 antagonist. *Nature Chemical Biology*, *5*(6), 421–427. https://doi.org/10.1038/nchembio.168

Dhillion, P., Wallace, K., Herse, F., Scott, J., Wallukat, G., Heath, J., … LaMarca, B. (2012). IL-17-mediated oxidative stress is an important stimulator of AT1-AA and hypertension during pregnancy. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *303*(4), R353–R358. https://doi.org/10.1152/ajpregu.00051.2012

Di Santo, S., Malek, A., Sager, A., Andres, A. C., & Schneider, H. (2003). Trophoblast viability in perfused term placental tissue and explant cultures limited to 7-24 hours. *Placenta*, *24*(8–9), 882–894. https://doi.org/10.1016/S0143-4004(03)00142-5

Di Simone, N., Di Nicuolo, F., Marzioni, D., Castellucci, M., Sanguinetti, M., D'Ippolito, S., & Caruso, A. (2009). Resistin modulates glucose uptake and glucose transporter-1 (GLUT-1) expression in trophoblast cells. *Journal of Cellular and Molecular Medicine*, *13*(2), 388–397. https://doi.org/10.1111/j.1582-4934.2008.00337.x

Diamanti-Kandarakis, E., Bourguignon, J.-P., Giudice, L. C., Hauser, R., Prins, G. S., Soto, A. M., … Gore, A. C. (2009). Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocrine Reviews*, *30*(4), 293–342. https://doi.org/10.1210/er.2009-0002

Dodson, R. E., Nishioka, M., Standley, L. J., Perovich, L. J., Brody, J. G., & Rudel, R. A. (2012). Endocrine disruptors and asthma-associated chemicals in consumer products. *Environmental Health Perspectives*, *120*(7), 935–43. https://doi.org/10.1289/ehp.1104052

Dolznig, H., Rupp, C., Puri, C., Haslinger, C., Schweifer, N., Wieser, E., … Garin-Chesa, P. (2011). Modeling colon adenocarcinomas in vitro a 3D co-culture system induces cancer-relevant pathways upon tumor cell and stromal fibroblast interaction. *The American Journal of Pathology*, *179*(1), 487–501. https://doi.org/10.1016/j.ajpath.2011.03.015

Domansky, K., Inman, W., Serdy, J., Dash, A., Lim, M. H. M., & Griffith, L. G. (2010). Perfused multiwell plate for 3D liver tissue engineering. *Lab on a Chip*, *10*(1), 51–8. https://doi.org/10.1039/b913221j

Donaghue, C., Westley, B. R., & May, F. E. B. (1999). Selective Promoter Usage of the Human Estrogen Receptor-α Gene and Its Regulation by Estrogen. *Molecular Endocrinology*, *13*(11), 1934–1950. https://doi.org/10.1210/mend.13.11.0366

Dong, R., Zhou, T., Chen, J., Zhang, M., Zhang, H., Wu, M., … Chen, B. (2017). Gender- and Age-Specific Relationships Between Phthalate Exposures and Obesity in Shanghai Adults. *Archives of Environmental Contamination and Toxicology*, *73*(3), 431–441. https://doi.org/10.1007/s00244-017-0441-6

Douglas, G. C., VandeVoort, C. A., Kumar, P., Chang, T.-C., & Golos, T. G. (2009). Trophoblast stem cells: models for investigating trophectoderm differentiation and placental development. *Endocrine Reviews*, *30*(3), 228–40. https://doi.org/10.1210/er.2009-0001

Duan, Y., Wang, L., Han, L., Wang, B., Sun, H., Chen, L., … Luo, Y. (2017). Exposure to phthalates in patients with diabetes and its association with oxidative stress, adiponectin, and inflammatory cytokines. *Environment International*, *109*, 53–63. https://doi.org/10.1016/j.envint.2017.09.002

Dutta-Roy, A. K. (2000). Transport mechanisms for long-chain polyunsaturated fatty acids in the human placenta. *Am J Clin Nutr*, *71*(1 Suppl), 315S–22S. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10617989

Dziennis, S., Yang, D., Cheng, J., Anderson, K. A., Alkayed, N. J., Hurn, P. D., & Lein, P. J. (2008). Developmental exposure to polychlorinated biphenyls influences stroke outcome in adult rats. *Environmental Health Perspectives*, *116*(4), 474–80. https://doi.org/10.1289/ehp.10828

Edlow, A. G., Chen, M., Smith, N. A., Lu, C., & McElrath, T. F. (2012). Fetal bisphenol A exposure: Concentration of conjugated and unconjugated bisphenol A in amniotic fluid in the second and third trimesters. *Reproductive Toxicology*, *34*(1), 1–7. https://doi.org/10.1016/j.reprotox.2012.03.009

Ehrlich, S., Williams, P. L., Missmer, S. A., Flaws, J. A., Ye, X., Calafat, A. M., … Hauser, R. (2012). Urinary bisphenol A concentrations and early reproductive health outcomes among women undergoing IVF. *Human Reproduction*, *27*(12), 3583–3592. https://doi.org/10.1093/humrep/des328

Ejaredar, M., Nyanza, E. C., Eycke, K. Ten, & Dewey, D. (2015). Phthalate exposure and childrens neurodevelopment: A systematic review. *Environmental Research*, *142*, 51–60. https://doi.org/10.1016/j.envres.2015.06.014

Eladak, S., Moison, D., Guerquin, M.-J., Matilionyte, G., Kilcoyne, K., N'Tumba-Byn, T., … Habert, R. (2018). Effects of environmental Bisphenol A exposures on germ cell development and Leydig cell function in the human fetal testis. *PLOS ONE*, *13*(1), e0191934. https://doi.org/10.1371/journal.pone.0191934

Engel, S. M., Miodovnik, A., Canfield, R. L., Zhu, C., Silva, M. J., Calafat, A. M., & Wolff, M. S. (2010). Prenatal Phthalate Exposure Is Associated with Childhood Behavior and Executive Functioning. *Environmental Health Perspectives*, *118*(4), 565–571. https://doi.org/10.1289/ehp.0901470

Erler, C., & Novak, J. (2010). Bisphenol A Exposure: Human Risk and Health Policy Background of Bisphenol A. *Journal of Pediatric Nursing*, *25*, 400–407. https://doi.org/10.1016/j.pedn.2009.05.006

Erol, O., Süren, D., Ellidağ, H. Y., Bülbül, G. A., Derbent, A. U., Elal, R., … Yılmaz, N. (2016). Serum level and placental expression of resistin in pregnancies complicated by preeclampsia: relationship with disease severity. *Clinical and Experimental Obstetrics & Gynecology*, *43*(4), 516–521. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/29734539

Esch, M. B., Sung, J. H., Yang, J., Yu, C., Yu, J., March, J. C., & Shuler, M. L. (2012). On chip porous polymer membranes for integration of gastrointestinal tract epithelium with microfluidic “body-on-a-chip” devices. *Biomedical Microdevices*, *14*(5), 895–906. https://doi.org/10.1007/s10544-012-9669-0

Eskenazi, B., Rauch, S. A., Tenerelli, R., Huen, K., Holland, N. T., Lustig, R. H., … Harley, K. G. (2017). In utero and childhood DDT, DDE, PBDE and PCBs exposure and sex hormones in adolescent boys: The CHAMACOS study. *International Journal of Hygiene and Environmental Health*, *220*(2), 364–372. https://doi.org/10.1016/j.ijheh.2016.11.001

Etienne-Manneville, S., & Hall, A. (2002). Rho GTPases in cell biology. *Nature*, *420*(6916), 629–635. https://doi.org/10.1038/nature01148

Evain-Brion, D., & Malassine, A. (2003). Human placenta as an endocrine organ. *Growth Hormone & IGF Research : Official Journal of the Growth Hormone Research Society and the International IGF Research Society*, *13 Suppl A*, S34-7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12914725

Evangelou, E., Ntritsos, G., Chondrogiorgi, M., Kavvoura, F. K., Hernández, A. F., Ntzani, E. E., & Tzoulaki, I. (2016). Exposure to pesticides and diabetes: A systematic review and meta-analysis. *Environment International*, *91*, 60–68. https://doi.org/10.1016/j.envint.2016.02.013

Everett, C. J., Frithsen, I. L., Diaz, V. A., Koopman, R. J., Simpson, W. M., & Mainous Iii, A. G. (2007). Association of a polychlorinated dibenzo-p-dioxin, a polychlorinated biphenyl, and DDT with diabetes in the 1999–2002 National Health and Nutrition Examination Survey. *Environmental Research*, *103*, 413–418. https://doi.org/10.1016/j.envres.2006.11.002

Fan, S., Liao, Y., Liu, C., Huang, Q., Liang, H., Ai, B., … Zhou, S. (2017a). Estrogen promotes tumor metastasis via estrogen receptor beta-mediated regulation of matrix-metalloproteinase-2 in non-small cell lung cancer. *Oncotarget*, *8*(34), 56443–56459. https://doi.org/10.18632/oncotarget.16992

Fan, S., Liao, Y., Liu, C., Huang, Q., Liang, H., Ai, B., … Zhou, S. (2017b). Estrogen promotes tumor metastasis via estrogen receptor beta-mediated regulation of matrix-metalloproteinase-2 in non-small cell lung cancer. *Oncotarget*, *8*(34), 56443–56459. https://doi.org/10.18632/oncotarget.16992

Fang, H., Tong, W., Perkins, R., Soto, A. M., Prechtl, N. V, & Sheehan, D. M. (2000). Quantitative comparisons of in vitro assays for estrogenic activities. *Environmental Health Perspectives*, *108*(8), 723–9. Retrieved from

http://www.ncbi.nlm.nih.gov/pubmed/10964792

Faroon, O., & Ruiz, P. (2015). Polychlorinated biphenyls: New evidence from the last decade. *Toxicology and Industrial Health*. https://doi.org/10.1177/0748233715587849

Fatima, S. S., Alam, F., Chaudhry, B., & Khan, T. A. (2017). Elevated levels of chemerin, leptin, and interleukin-18 in gestational diabetes mellitus. *Journal of Maternal-Fetal and Neonatal Medicine*, *30*(9), 1023–1028. https://doi.org/10.1080/14767058.2016.1199671

Favata, M. F., Horiuchi, K. Y., Manos, E. J., Daulerio, A. J., Stradley, D. A., Feeser, W. S., … Trzaskos, J. M. (1998). Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *The Journal of Biological Chemistry*, *273*(29), 18623–32. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9660836

Feng, X., Zhou, L., Mao, X., Tong, C., Chen, X., Zhao, D., … Zhang, H. (2017). Association of a reduction of G-protein coupled receptor 30 expression and the pathogenesis of preeclampsia. *Molecular Medicine Reports*, *16*(5), 5997–6003. https://doi.org/10.3892/mmr.2017.7341

Ferguson, K. K., McElrath, T. F., Cantonwine, D. E., Mukherjee, B., & Meeker, J. D. (2015). Phthalate metabolites and bisphenol-A in association with circulating angiogenic biomarkers across pregnancy. *Placenta*, *36*(6), 699–703. https://doi.org/10.1016/j.placenta.2015.04.002

Ferguson, K. K., Mcelrath, T. F., Ko, Y.-A., Mukherjee, B., & Meeker, J. D. (2014). Variability in urinary phthalate metabolite levels across pregnancy and sensitive windows of exposure for the risk of preterm birth. https://doi.org/10.1016/j.envint.2014.05.016

Filardo, E. J., Quinn, J. A., Bland, K. I., & Frackelton, A. R. (2000). Estrogen-Induced Activation of Erk-1 and Erk-2 Requires the G Protein-Coupled Receptor Homolog, GPR30, and Occurs via *Trans* -Activation of the Epidermal Growth Factor Receptor through Release of HB-EGF. *Molecular Endocrinology*, *14*(10), 1649–1660. https://doi.org/10.1210/mend.14.10.0532

Fischbach, C., Kong, H. J., Hsiong, S. X., Evangelista, M. B., Yuen, W., & Mooney, D. J. (2009). Cancer cell angiogenic capability is regulated by 3D culture and integrin engagement. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(2), 399–404. https://doi.org/10.1073/pnas.0808932106

Fitri, L. E., Sardjono, T. W., Rahmah, Z., Siswanto, B., Handono, K., & Dachlan, Y. P. (2015). Low Fetal Weight is Directly Caused by Sequestration of Parasites and Indirectly by IL-17 and IL-10 Imbalance in the Placenta of Pregnant Mice with Malaria. *The Korean Journal of Parasitology*, *53*(2), 189–196. https://doi.org/10.3347/kjp.2015.53.2.189

Fromme, H., Gruber, L., Seckin, E., Raab, U., Zimmermann, S., Kiranoglu, M., … the HBMnet, for. (2011). Phthalates and their metabolites in breast milk — Results from the Bavarian Monitoring of Breast Milk (BAMBI). https://doi.org/10.1016/j.envint.2011.02.008

Fujimoto, J., Nakagawa, Y., Toyoki, H., Sakaguchi, H., Sato, E., & Tamaya, T. (2005). Estrogen-related receptor expression in placenta throughout gestation. *The Journal of Steroid Biochemistry and Molecular Biology*, *94*(1–3), 67–9. https://doi.org/10.1016/j.jsbmb.2004.12.030

Galardi, S., Petretich, M., Pinna, G., D'Amico, S., Loreni, F., Michienzi, A., … Ciafrè, S. A. (2016). CPEB1 restrains proliferation of Glioblastoma cells through the regulation of p27(Kip1) mRNA translation. *Scientific Reports*, *6*, 25219. https://doi.org/10.1038/srep25219

Ganer Herman, H., Barber, E., Gasnier, R., Gindes, L., Bar, J., Schreiber, L., & Kovo, M. (2018). Placental pathology and neonatal outcome in small for gestational age pregnancies with and without abnormal umbilical artery Doppler flow. *European Journal of Obstetrics & Gynecology and Reproductive Biology*, *222*, 52–56. https://doi.org/10.1016/j.ejogrb.2018.01.009

García-Arévalo, M., Alonso-Magdalena, P., Servitja, J.-M., Boronat-Belda, T., Merino, B., Villar-Pazos, S., … Nadal, A. (n.d.). Maternal Exposure to Bisphenol-A During Pregnancy Increases Pancreatic ᴺʟ-Cell Growth During Early Life in Male Mice Offspring. *I.Q.* https://doi.org/10.1210/en.2016-1390

Gathiram, P., & Moodley, J. (2016). Pre-eclampsia: its pathogenesis and pathophysiolgy. *Cardiovascular Journal of Africa*, *27*(2), 71–78. https://doi.org/10.5830/CVJA-2016-009

Ghantous, C. M., Kobeissy, F. H., Soudani, N., Rahman, F. A., Al-Hariri, M., Itani, H. A., … Zeidan, A. (2015). Mechanical stretch-induced vascular hypertrophy occurs through modulation of leptin synthesis-mediated ROS formation and GATA-4 nuclear translocation. *Frontiers in Pharmacology*, *6*, 240. https://doi.org/10.3389/fphar.2015.00240

Ghasemi, A., Isaac Hashemy, S., Aghaei, M., & Panjehpour, M. (2017). RhoA/ROCK pathway mediates leptin-induced uPA expression to promote cell invasion in ovarian cancer cells. https://doi.org/10.1016/j.cellsig.2017.01.020

Gilmartin, A. B. H., Ural, S. H., & Repke, J. T. (2008). Gestational diabetes mellitus. *Reviews in Obstetrics & Gynecology*, *1*(3), 129–34. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/19015764

Giusti, R. M., Iwamoto, K., & Hatch, E. E. (1995). Diethylstilbestrol Revisited: A Review of the Long-Term Health Effects. *Annals of Internal Medicine*, *122*(10), 778. https://doi.org/10.7326/0003-4819-122-10-199505150-00008

Glazier, J. D., & Jansson, T. (2004). Placental transport in early pregnancy--a workshop report. *Placenta*, *25 Suppl A*, S57-9. https://doi.org/10.1016/j.placenta.2004.01.022

Goodson, W. H., Luciani, M. G., Sayeed, S. A., Jaffee, I. M., Moore, D. H., & Dairkee, S. H. (2011). Activation of the mTOR pathway by low levels of xenoestrogens in breast epithelial cells from high-risk women. *Carcinogenesis*, *32*(11), 1724–1733. https://doi.org/10.1093/carcin/bgr196

Gore, A. C., Crews, D., Doan, L. L., Merrill, M. La, Patisaul, H., & Zota, A. (2014).

Introduction to endocrine disrupting Chemicals (EDCs. A Guide fir public interest organizations and policy-makers. Retrieved from https://www.endocrine.org/-/media/endosociety/files/advocacy-and-outreach/important-documents/introduction-to-endocrine-disrupting-chemicals.pdf

Grassi, T. F., da Silva, G. N., Bidinotto, L. T., Rossi, B. F., Quinalha, M. M., Kass, L., … Barbisan, L. F. (2016). Global gene expression and morphological alterations in the mammary gland after gestational exposure to bisphenol A, genistein and indole-3-carbinol in female Sprague-Dawley offspring. *Toxicology and Applied Pharmacology*, *303*, 101–109. https://doi.org/10.1016/j.taap.2016.05.004

Gude, N. M., Roberts, C. T., Kalionis, B., & King, R. G. (2004). Growth and function of the normal human placenta. *Thrombosis Research*, *114*(5–6 SPEC. ISS.), 397–407. https://doi.org/10.1016/j.thromres.2004.06.038

Guida, M., Troisi, J., Ciccone, C., Granozio, G., Cosimato, C., Sardo, A. D. S., … Di Carlo, C. (2015). Bisphenol A and congenital developmental defects in humans. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*, *774*, 33–39. https://doi.org/10.1016/j.mrfmmm.2015.02.007

Gundert-Remy, U., Bodin, J., Bosetti, C., FitzGerald, R., Hanberg, A., Hass, U., … Castoldi, A. F. (2017). Bisphenol A (BPA) hazard assessment protocol. *EFSA Supporting Publications*, *14*(12). https://doi.org/10.2903/sp.efsa.2017.EN-1354

Guo, Z., Qiu, H., Wang, L., Wang, L., Wang, C., Chen, M., & Zuo, Z. (2017). Association of serum organochlorine pesticides concentrations with reproductive hormone levels and polycystic ovary syndrome in a Chinese population. https://doi.org/10.1016/j.chemosphere.2016.12.127

Guttmacher, A. E., Maddox, Y. T., & Spong, C. Y. (2014). The Human Placenta Project: placental structure, development, and function in real time. *Placenta*, *35*(5), 303–4. https://doi.org/10.1016/j.placenta.2014.02.012

Guzmán-Bárcenas, J., Hernández, J. A., Arias-Martínez, J., Baptista-González, H., Ceballos-Reyes, G., & Irles, C. (2016). Estimation of umbilical cord blood leptin and insulin based on anthropometric data by means of artificial neural network approach: identifying key maternal and neonatal factors. *BMC Pregnancy and Childbirth*, *16*(1), 179. https://doi.org/10.1186/s12884-016-0967-z

Haggarty, P. (2002). Placental regulation of fatty acid delivery and its effect on fetal growth--a review. *Placenta*, *23 Suppl A*, S28–S38. https://doi.org/10.1053/plac.2002.0791

Hahlin, M., Wallin, A., Sjöblom, P., & Lindblom, B. (1990). Single progesterone assay for early recognition of abnormal pregnancy. *Human Reproduction*, *5*(5), 622–626. https://doi.org/10.1093/oxfordjournals.humrep.a137157

Haque, I., Ghosh, A., Acup, S., Banerjee, S., Dhar, K., Ray, A., … Banerjee, S. K. (2018). Leptin-induced ER-α-positive breast cancer cell viability and migration is mediated by suppressing CCN5-signaling via activating JAK/AKT/STAT-pathway. *BMC Cancer*, *18*(1), 99. https://doi.org/10.1186/s12885-018-3993-6

Hara, C. de C. P., França, E. L., Fagundes, D. L. G., de Queiroz, A. A., Rudge, M. V.

C., Honorio-França, A. C., & Calderon, I. de M. P. (2016). Characterization of Natural Killer Cells and Cytokines in Maternal Placenta and Fetus of Diabetic Mothers. *Journal of Immunology Research*, *2016*, 7154524. https://doi.org/10.1155/2016/7154524

Harlid, S., Adgent, M., Jefferson, W. N., Panduri, V., Umbach, D. M., Xu, Z., … Taylor, J. A. (2017). Soy Formula and Epigenetic Modifications: Analysis of Vaginal Epithelial Cells from Infant Girls in the IFED Study. *Environmental Health Perspectives*, *125*(3), 447–452. https://doi.org/10.1289/EHP428

Hart, R. J., Doherty, D. A., Keelan, J. A., Minaee, N. S., Thorstensen, E. B., Dickinson, J. E., … Handelsman, D. J. (2018). The impact of antenatal Bisphenol A exposure on male reproductive function at 20–22 years of age. *Reproductive BioMedicine Online*, *36*(3), 340–347. https://doi.org/10.1016/j.rbmo.2017.11.009

Hart, R., Sloboda, D. M., Doherty, D. A., Norman, R. J., Atkinson, H. C., Newnham, J. P., … Hickey, M. (2010). Circulating maternal testosterone concentrations at 18 weeks of gestation predict circulating levels of antimüllerian hormone in adolescence: a prospective cohort study. *Fertility and Sterility*, *94*(4), 1544–1547. https://doi.org/10.1016/j.fertnstert.2009.12.060

Heldring, N., Pike, A., Andersson, S., Matthews, J., Cheng, G., Treuter, E., … Stro, A. (2007). Estrogen Receptors : How Do They Signal and What Are Their Targets. *Physiology Review*, *87*, 905–931. https://doi.org/10.1152/physrev.00026.2006.

Hewlett, M., Chow, E., Aschengrau, A., & Mahalingaiah, S. (2017). Prenatal Exposure to Endocrine Disruptors: A Developmental Etiology for Polycystic Ovary Syndrome. https://doi.org/10.1177/1933719116654992

Hijazi, A., Guan, H., Cernea, M., & Yang, K. (2015). Prenatal exposure to bisphenol A disrupts mouse fetal lung development. *The FASEB Journal*, *29*(12), 4968–4977. https://doi.org/10.1096/fj.15-270942

Hill, D. J. (2018). Placental control of metabolic adaptations in the mother for an optimal pregnancy outcome. What goes wrong in gestational diabetes? *Placenta*. https://doi.org/10.1016/j.placenta.2018.01.002

Hodges, C. A., Ilagan, A., Jennings, D., Keri, R., Nilson, J., & Hunt, P. A. (2002). Experimental evidence that changes in oocyte growth influence meiotic chromosome segregation. *Human Reproduction*, *17*(5), 1171–1180. https://doi.org/10.1093/humrep/17.5.1171

Hoepner, L. A., Whyatt, R. M., Widen, E. M., Hassoun, A., Oberfield, S. E., Mueller, N. T., … Rundle, A. G. (2016). Bisphenol A and Adiposity in an Inner-City Birth Cohort. *Environmental Health Perspectives*, *124*(10), 1644–1650. https://doi.org/10.1289/EHP205

Howdeshell, K. L., vom Saal, F. S., Hotchkiss, A. K., Thayer, K. A., & Vandenbergh, J. G. (1999). Environmental toxins: Exposure to bisphenol A advances puberty. *Nature*, *401*(6755), 763–764. https://doi.org/10.1038/44517

Hu, L., Zaloudek, C., Mills, G. B., Gray, J., & Jaffe, R. B. (2000). In vivo and in vitro ovarian carcinoma growth inhibition by a phosphatidylinositol 3-kinase inhibitor

(LY294002). *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *6*(3), 880–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10741711

Hu, R., Hu, F., Xie, X., Wang, L., Li, G., Qiao, T., … Xiao, H. (2016). TMEM45B, up-regulated in human lung cancer, enhances tumorigenicity of lung cancer cells. *Tumor Biology*, *37*(9), 12181–12191. https://doi.org/10.1007/s13277-016-5063-5

Huang, D.-Y., Zheng, C.-C., Pan, Q., Wu, S.-S., Su, X., Li, L., … Sun, Z.-Y. (2018). Oral exposure of low-dose bisphenol A promotes proliferation of dorsolateral prostate and induces epithelial-mesenchymal transition in aged rats. *Scientific Reports*, *8*(1), 490. https://doi.org/10.1038/s41598-017-18869-8

Huang, D., Wu, J., Su, X., Yan, H., & Sun, Z. (2017). Effects of low dose of bisphenol A on the proliferation and mechanism of primary cultured prostate epithelial cells in rodents. *Oncology Letters*, *14*(3), 2635–2642. https://doi.org/10.3892/ol.2017.6469

Huang, H., Kong, D., Byun, K. H., Ye, C., Koda, S., Lee, D. H., … Kim, Y.-B. (2012). Rho-kinase regulates energy balance by targeting hypothalamic leptin receptor signaling. *Nature Neuroscience*, *15*(10), 1391–8. https://doi.org/10.1038/nn.3207

Hughes, C. S., Postovit, L. M., & Lajoie, G. A. (2010). Matrigel: A complex protein mixture required for optimal growth of cell culture. *PROTEOMICS*, *10*(9), 1886–1890. https://doi.org/10.1002/pmic.200900758

Huh, D., Matthews, B. D., Mammoto, A., Montoya-Zavala, M., Hsin, H. Y., & Ingber, D. E. (2010). Reconstituting organ-level lung functions on a chip. *Science (New York, N.Y.)*, *328*(5986), 1662–8. https://doi.org/10.1126/science.1188302

Hui, L., Li, H., Lu, G., Chen, Z., Sun, W., Shi, Y., … Wu, Y. (2018). Low Dose of Bisphenol A Modulates Ovarian Cancer Gene Expression Profile and Promotes Epithelial to Mesenchymal Transition Via Canonical Wnt Pathway. *Toxicological Sciences*. https://doi.org/10.1093/toxsci/kfy107

Hunt, P. A., Koehler, K. E., Susiarjo, M., Hodges, C. A., Ilagan, A., Voigt, R. C., … Hassold, T. J. (2003). Bisphenol A Exposure Causes Meiotic Aneuploidy in the Female Mouse are the leading cause of miscarriage, congenital defects, and mental retardation [1]. Because almost all such an- euploidy derives from meiotic errors, considerable effort. *Current Biology*, *13*, 546–553. https://doi.org/10.1016/S

Huo, W., Xia, W., Wan, Y., Zhang, B., Zhou, A., Zhang, Y., … Xu, S. (2015). Maternal urinary bisphenol A levels and infant low birth weight: A nested case-control study of the Health Baby Cohort in China. *Environment International*, *85*, 96–103. https://doi.org/10.1016/j.envint.2015.09.005

Huppertz, B., Frank, H. G., Kingdom, J. C., Reister, F., & Kaufmann, P. (1998). Villous cytotrophoblast regulation of the syncytial apoptotic cascade in the human placenta. *Histochemistry and Cell Biology*, *110*(5), 495–508. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9826129

Ikezuki, Y., Tsutsumi, O., Takai, Y., Kamei, Y., & Taketani, Y. (2002). Determination

of bisphenol A concentrations in human biological fluids reveals significant early prenatal exposure. *Human Reproduction*, *17*(11), 2839–2841. https://doi.org/10.1093/humrep/17.11.2839

Ishii, I., Friedman, B., Ye, X., Kawamura, S., McGiffert, C., Contos, J. J., … Chun, J. (2001). Selective loss of sphingosine 1-phosphate signaling with no obvious phenotypic abnormality in mice lacking its G protein-coupled receptor, LP(B3)/EDG-3. *The Journal of Biological Chemistry*, *276*(36), 33697–704. https://doi.org/10.1074/jbc.M104441200

Ivanciuc, T., Ivanciuc, O., & Klein, D. J. (2006). Modeling the bioconcentration factors and bioaccumulation factors of polychlorinated biphenyls with posetic quantitative super-structure/activity relationships (QSSAR). *Molecular Diversity*, *10*(2), 133–145. https://doi.org/10.1007/s11030-005-9003-3

Ivanova, M. M., Mazhawidza, W., Dougherty, S. M., & Klinge, C. M. (2010). Sex differences in estrogen receptor subcellular location and activity in lung adenocarcinoma cells. *American Journal of Respiratory Cell and Molecular Biology*, *42*(3), 320–30. https://doi.org/10.1165/rcmb.2009-0059OC

Jaffe, R., Jauniaux, E., & Hustin, J. (1997). Maternal circulation in the first-trimester human placenta - Myth or reality? *American Journal of Obstetrics and Gynecology*, *176*(3), 695–705. https://doi.org/10.1016/S0002-9378(97)70572-6

Jaimes, R., Swiercz, A., Sherman, M., Muselimyan, N., Marvar, P. J., & Posnack, N. G. (2017). Plastics and cardiovascular health: phthalates may disrupt heart rate variability and cardiovascular reactivity. *American Journal of Physiology-Heart and Circulatory Physiology*, *313*(5), H1044–H1053. https://doi.org/10.1152/ajpheart.00364.2017

James, J. L., Carter, A. M., & Chamley, L. W. (2012). Human placentation from nidation to 5 weeks of gestation. Part II: Tools to model the crucial first days. *Placenta*, *33*, 335–342. https://doi.org/10.1016/j.placenta.2012.01.019

Jang, K.-J., Mehr, A. P., Hamilton, G. A., McPartlin, L. A., Chung, S., Suh, K.-Y., & Ingber, D. E. (2013). Human kidney proximal tubule-on-a-chip for drug transport and nephrotoxicity assessment. *Integrative Biology : Quantitative Biosciences from Nano to Macro*, *5*(9), 1119–29. https://doi.org/10.1039/c3ib40049b

Jauniaux, E., Gulbis, B., & Burton, G. J. (2003). Physiological implications of the materno-fetal oxygen gradient in human early pregnancy. *Reproductive BioMedicine Online*, *7*(2), 250–253. https://doi.org/10.1016/S1472-6483(10)61760-9

JeBailey, L., Wanono, O., Niu, W., Roessler, J., Rudich, A., & Klip, A. (2007). Ceramide- and oxidant-induced insulin resistance involve loss of insulin-dependent Rac-activation and actin remodeling in muscle cells. *Diabetes*, *56*(2), 394–403. https://doi.org/10.2337/db06-0823

Jeon, E. J., Hong, S. Y., & Lee, J. H. (2017). Adipokines and Insulin Resistance According to Characteristics of Pregnant Women with Gestational Diabetes Mellitus. *Diabetes & Metabolism Journal*, *41*(6), 457–465. https://doi.org/10.4093/dmj.2017.41.6.457

Jeong, J. S., Nam, K. T., Lee, B., Pamungkas, A. D., Song, D., Kim, M., … Lim, K.-M. (2017). Low-Dose Bisphenol A Increases Bile Duct Proliferation in Juvenile Rats: A Possible Evidence for Risk of Liver Cancer in the Exposed Population? *Biomolecules & Therapeutics*, *25*(5), 545–552. https://doi.org/10.4062/biomolther.2017.148

Jia, M., Dahlman-Wright, K., & Gustafsson, J.-Å. (2015). Estrogen receptor alpha and beta in health and disease. *Best Practice & Research Clinical Endocrinology & Metabolism*, *29*(4), 557–568. https://doi.org/10.1016/j.beem.2015.04.008

Jia, Y., Ying, X., Zhou, J., Chen, Y., Luo, X., Xie, S., … Wang, L. (2018). The novel KLF4/PLAC8 signaling pathway regulates lung cancer growth. *Cell Death & Disease*, *9*(6), 603. https://doi.org/10.1038/s41419-018-0580-3

Jiang, L.-G., Cheng, L.-Y., Kong, S.-H., Yang, Y., Shen, Y.-J., Chen, C., … Chao, L. (2017). Toxic effects of polychlorinated biphenyls (Aroclor 1254) on human sperm motility. *Asian Journal of Andrology*, *19*(5), 561–566. https://doi.org/10.4103/1008-682X.186876

Jiang, S.-W., Zhou, W., Wang, J., Little, L. M., Leaphart, L., Jay, J., … Li, J. (2018). Gene expression patterns associated with human placental trophoblast differentiation. *Clinica Chimica Acta*. https://doi.org/10.1016/j.cca.2018.01.012

Johansson, H. K. L., Jacobsen, P. R., Hass, U., Svingen, T., Vinggaard, A. M., Isling, L. K., … Boberg, J. (2016). Perinatal exposure to mixtures of endocrine disrupting chemicals reduces female rat follicle reserves and accelerates reproductive aging. *Reproductive Toxicology*, *61*, 186–194. https://doi.org/10.1016/j.reprotox.2016.03.045

Kagami, M., Kurosawa, K., Miyazaki, O., Ishino, F., Matsuoka, K., & Ogata, T. (2015). Comprehensive clinical studies in 34 patients with molecularly defined UPD(14)pat and related conditions (Kagami-Ogata syndrome). *European Journal of Human Genetics : EJHG*, *23*(11), 1488–98. https://doi.org/10.1038/ejhg.2015.13

Kaistha, B. P., Lorenz, H., Schmidt, H., Sipos, B., Pawlak, M., Gierke, B., … Buchholz, M. (2016). PLAC8 localizes to the inner plasma membrane of pancreatic cancer cells and regulates cell growth and disease progression through critical cell-cycle regulatory pathways. *Cancer Research*, *76*(1), 96–107. https://doi.org/10.1158/0008-5472.CAN-15-0216

Kampa, M., Pelekanou, V., Notas, G., Stathopoulos, E. N., & Castanas, E. (2013). The estrogen receptor: Two or more molecules, multiple variants, diverse localizations, signaling and functions. Are we undergoing a paradigm-shift as regards their significance in breast cancer? *Hormones*, *12*(1), 69–85.

Karimian, E., Chagin, A. S., & Sävendahl, L. (2011). Genetic regulation of the growth plate. *Frontiers in Endocrinology*, *2*, 113. https://doi.org/10.3389/fendo.2011.00113

Kasper-Sonnenberg, M., Wittsiepe, J., Wald, K., Koch, H. M., & Wilhelm, M. (2017). Pre-pubertal exposure with phthalates and bisphenol A and pubertal development. *PloS One*, *12*(11), e0187922.

https://doi.org/10.1371/journal.pone.0187922

Kelly, A. C., Rodgers, A., Dong, K.-W., Barrezueta, N. X., Blum, M., & L., R. J. (1991). Gonadotropin-Releasing Hormone and Chorionic Gonadotropin Gene Expression in Human Placental Development. *DNA and Cell Biology*, *10*(6), 411–421. https://doi.org/10.1089/dna.1991.10.411

Khong, T. Y., & Yee, K. T. (1989). Pathology of intrauterine growth retardation. *American Journal of Reproductive Immunology (New York, N.Y. : 1989)*, *21*(3–4), 132–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2701165

Kim, B.-N., Cho, S.-C., Kim, Y., Shin, M.-S., Yoo, H.-J., Kim, J.-W., … Hong, Y.-C. (2009). Phthalates Exposure and Attention-Deficit/Hyperactivity Disorder in School-Age Children. *Biological Psychiatry*, *66*(10), 958–963. https://doi.org/10.1016/j.biopsych.2009.07.034

Kim, K.-S., Lee, Y.-M., Kim, S. G., Lee, I.-K., Lee, H.-J., Kim, J.-H., … Lee, D.-H. (2014). Associations of organochlorine pesticides and polychlorinated biphenyls in visceral vs. subcutaneous adipose tissue with type 2 diabetes and insulin resistance. https://doi.org/10.1016/j.chemosphere.2013.09.066

Kim, S. C., Park, M.-N., Lee, Y. J., Joo, J. K., & An, B.-S. (2016). Interaction of steroid receptor coactivators and estrogen receptors in the human placenta. *Journal of Molecular Endocrinology*, *56*(3), 239–247. https://doi.org/10.1530/JME-15-0248

Kim, Y., Ha, E., Kim, E., Park, H., Ha, M., Kim, J., … Kim, B. (2011). Prenatal Exposure to Phthalates and Infant Development at 6 Months: Prospective Mothers and Children's Environmental Health (MOCEH) Study. *Environmental Health Perspectives*, *119*(10), 1495–1500. https://doi.org/10.1289/ehp.1003178

Kimber, I., & Dearman, R. J. (2010). An assessment of the ability of phthalates to influence immune and allergic responses. *Toxicology*, *271*, 73–82. https://doi.org/10.1016/j.tox.2010.03.020

King, A., Thomas, L., & Bischof, P. (2000). Cell culture models of trophoblast II: Trophoblast cell lines - A workshop report. *Placenta*, *21*(SUPPL.1), 113–119. https://doi.org/10.1053/plac.1999.0526

Kingdom, J., Huppertz, B., Seaward, G., & Kaufmann, P. (2000). Development of the placental villous tree and its consequences for fetal growth. *European Journal of Obstetrics Gynecology and Reproductive Biology*, *92*(1), 35–43. https://doi.org/10.1016/S0301-2115(00)00423-1

Koch, C. A., & Diamanti-Kandarakis, E. (2015). Introduction to Endocrine Disrupting Chemicals – is it time to act? *Reviews in Endocrine and Metabolic Disorders*, *16*(4), 269–270. https://doi.org/10.1007/s11154-016-9338-3

Koike, E., Yanagisawa, R., Win-Shwe, T.-T., & Takano, H. (2018). Exposure to low-dose bisphenol A during the juvenile period of development disrupts the immune system and aggravates allergic airway inflammation in mice. *International Journal of Immunopathology and Pharmacology*, *32*, 2058738418774897. https://doi.org/10.1177/2058738418774897

Kokkinos, M. I., Murthi, P., Wafai, R., Thompson, E. W., & Newgreen, D. F. (2010). Cadherins in the human placenta – epithelial–mesenchymal transition (EMT) and placental development. *Placenta*, *31*(9), 747–755. https://doi.org/10.1016/J.PLACENTA.2010.06.017

Kolluru, V., Pal, D., Papu John, A. M. S., Ankem, M. K., Freedman, J. H., & Damodaran, C. (2017). Induction of Plac8 promotes pro-survival function of autophagy in cadmium-induced prostate carcinogenesis. *Cancer Letters*, *408*, 121–129. https://doi.org/10.1016/j.canlet.2017.08.023

Kong, X., Yan, D., Sun, J., Wu, X., Mulder, H., Hua, X., & Ma, X. (2014). Glucagon-Like Peptide 1 Stimulates Insulin Secretion via Inhibiting RhoA/ROCK Signaling and Disassembling Glucotoxicity-Induced Stress Fibers. *Endocrinology*, *155*(12), 4676–4685. https://doi.org/10.1210/en.2014-1314

Krishnan, A. V, Stathis, P., Permuth, S. F., Tokes, L., & Feldman, D. (1993). Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology*, *132*(6). https://doi.org/10.1210/endo.132.6.8504731

Krueger, G. G., Langley, R. G., Leonardi, C., Yeilding, N., Guzzo, C., Wang, Y., … Lebwohl, M. (2007). A Human Interleukin-12/23 Monoclonal Antibody for the Treatment of Psoriasis. *New England Journal of Medicine*, *356*(6), 580–592. https://doi.org/10.1056/NEJMoa062382

Kudo, Y., Boyd, C. A. R., Kimura, H., Cook, P. R., Redman, C. W. G., & Sargent, I. L. (2003a). Quantifying the syncytialisation of human placental trophoblast BeWo cells grown in vitro. *Biochimica et Biophysica Acta*, *1640*(1), 25–31. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12676351

Kudo, Y., Boyd, C. A. R., Kimura, H., Cook, P. R., Redman, C. W. G., & Sargent, I. L. (2003b). Quantifying the syncytialisation of human placental trophoblast BeWo cells grown in vitro. *Biochimica et Biophysica Acta - Molecular Cell Research*, *1640*(1), 25–31. https://doi.org/10.1016/S0167-4889(03)00004-1

Kumar, A., Banerjee, A., Singh, D., Thakur, G., Kasarpalkar, N., Gavali, S., … Sachdeva, G. (2018). Estradiol: A Steroid with Multiple Facets. *Hormone and Metabolic Research*. https://doi.org/10.1055/s-0044-100920

Kurosawa, T., Hiroi, H., Tsutsumi, O., Ishikawa, T., Osuga, Y., Fujiwara, T., … Taketani, Y. (2002). The activity of bisphenol A depends on both the estrogen receptor subtype and the cell type. *Endocrine Journal*, *49*(4), 465–71. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12402979

Lakshmi Devi, C. K., & Raghupathy, N. S. (n.d.). The Histological Findings in Human Placenta at Different Gestational Ages. *IOSR Journal of Dental and Medical Sciences*, *6*(1), 2279–861. Retrieved from www.iosrjournals.org

Lamartiniere, C. A., Jenkins, S., Betancourt, A. M., Wang, J., & Russo, J. (2011). Exposure to the endocrine disruptor bisphenol A alters susceptibility for mammary cancer. *Hormone Molecular Biology and Clinical Investigation*, *5*(2), 45–52. https://doi.org/10.1515/HMBCI.2010.075

Lawson, C., Gieske, M., Murdoch, B., Ye, P., Li, Y., Hassold, T., & Hunt, P. A. (2011).

Gene Expression in the Fetal Mouse Ovary Is Altered by Exposure to Low Doses of Bisphenol A1. *Biology of Reproduction*, *84*(1), 79–86. https://doi.org/10.1095/biolreprod.110.084814

Le Dréan, G., Haure-Mirande, V., Ferrier, L., Bonnet, C., Hulin, P., De Coppet, P., & Segain, J.-P. (2014). Visceral adipose tissue and leptin increase colonic epithelial tight junction permeability via a RhoA-ROCK-dependent pathway. https://doi.org/10.1096/fj.13-234203

Leclerc, F., Dubois, M.-F., & Aris, A. (2014). Maternal, placental and fetal exposure to bisphenol A in women with and without preeclampsia. *Hypertension in Pregnancy*, *33*(3), 341–348. https://doi.org/10.3109/10641955.2014.892607

Lee, H.-R., Kim, T.-H., & Choi, K.-C. (2012). Functions and physiological roles of two types of estrogen receptors, ERα and ERβ, identified by estrogen receptor knockout mouse. *Laboratory Animal Research*, *28*(2), 71. https://doi.org/10.5625/lar.2012.28.2.71

Lee, H. J., Chattopadhyay, S., Gong, E.-Y., Ahn, R. S., & Lee, K. (2003). Antiandrogenic Effects of Bisphenol A and Nonylphenol on the Function of Androgen Receptor. *Toxicological Sciences*, *75*(1), 40–46. https://doi.org/10.1093/toxsci/kfg150

Lee, J. S., Romero, R., Han, Y. M., Kim, H. C., Kim, C. J., Hong, J.-S., & Huh, D. (2016). Placenta-on-a-chip: a novel platform to study the biology of the human placenta. *The Journal of Maternal-Fetal & Neonatal Medicine*, *29*(7), 1046–1054. https://doi.org/10.3109/14767058.2015.1038518

Lee, Y.-R., Park, J., Yu, H.-N., Kim, J.-S., Youn, H. J., & Jung, S. H. (2005). Up-regulation of PI3K/Akt signaling by 17β-estradiol through activation of estrogen receptor-α, but not estrogen receptor-β, and stimulates cell growth in breast cancer cells. *Biochemical and Biophysical Research Communications*, *336*(4), 1221–1226. https://doi.org/10.1016/j.bbrc.2005.08.256

Lee, Y., Wang, M. Y., Kakuma, T., Wang, Z. W., Babcock, E., McCorkle, K., … Unger, R. H. (2001). Liporegulation in diet-induced obesity. The antisteatotic role of hyperleptinemia. *The Journal of Biological Chemistry*, *276*(8), 5629–35. https://doi.org/10.1074/jbc.M008553200

Leguina-Ruzzi, A., Pereira, J., Pereira-Flores, K., Valderas, J. P., Mezzano, D., Velarde, V., & Sáez, C. G. (2015). Increased RhoA/Rho-Kinase Activity and Markers of Endothelial Dysfunction in Young Adult Subjects with Metabolic Syndrome. *Metabolic Syndrome and Related Disorders*, *13*(9), 373–380. https://doi.org/10.1089/met.2015.0061

Lelièvre, S. A., Kwok, T., & Chittiboyina, S. (2017). Architecture in 3D cell culture: An essential feature for in vitro toxicology. *Toxicology in Vitro*, *45*(March), 287–295. https://doi.org/10.1016/j.tiv.2017.03.012

Levin, E. R. (2005). Integration of the extranuclear and nuclear actions of estrogen. *Molecular Endocrinology (Baltimore, Md.)*, *19*(8), 1951–9. https://doi.org/10.1210/me.2004-0390

Li, C., Ma, H., Wang, Y., Cao, Z., Graves-Deal, R., Powell, A. E., … Coffey, R. J. (2014). Excess PLAC8 promotes an unconventional ERK2-dependent EMT in colon cancer. *The Journal of Clinical Investigation*, *124*(5), 2172–87. https://doi.org/10.1172/JCI71103

Li, D., Zhou, Z., Qing, D., He, Y., Wu, T., Miao, M., … Yuan, W. (2010). Occupational exposure to bisphenol-A (BPA) and the risk of Self-Reported Male Sexual Dysfunction. *Human Reproduction*, *25*(2), 519–527. https://doi.org/10.1093/humrep/dep381

Li, H. W., Cheung, A. N. Y., Tsao, S. W., Cheung, A. L. M., & O, W. S. (2003). Expression of e-cadherin and beta-catenin in trophoblastic tissue in normal and pathological pregnancies. *International Journal of Gynecological Pathology : Official Journal of the International Society of Gynecological Pathologists*, *22*(1), 63–70. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12496700

Li, J., Chen, Z., Zhou, X., Shi, S., Qi, H., Baker, P. N., & Zhang, H. (2016). Imbalance between proliferation and apoptosis-related impaired GPR30 expression is involved in preeclampsia. *Cell and Tissue Research*, *366*(2), 499–508. https://doi.org/10.1007/s00441-016-2466-y

Li, Q., Davila, J., Kannan, A., Flaws, J. A., Bagchi, M. K., & Bagchi, I. C. (2016). Chronic exposure to bisphenol a affects uterine function during early pregnancy in mice. *Endocrinology*, *157*(5), 1764–1774. https://doi.org/10.1210/en.2015-2031

Li, X. L., Dong, X., Xue, Y., Li, C. F., Gou, W. L., & Chen, Q. (2014). Increased expression levels of E-cadherin, cytokeratin 18 and 19 observed in preeclampsia were not correlated with disease severity. *Placenta*, *35*(8), 625–631. https://doi.org/10.1016/j.placenta.2014.04.010

Li, Y., Guo, W., Liu, S., Zhang, B., Yu, B.-B., Yang, B., … Feng, S.-Q. (2017). Silencing Transmembrane Protein 45B (TNEM45B) Inhibits Proliferation, Invasion, and Tumorigenesis in Osteosarcoma Cells. *Oncology Research Featuring Preclinical and Clinical Cancer Therapeutics*, *25*(6), 1021–1026. https://doi.org/10.3727/096504016X14821477992177

Li, Z., Cheng, L., Liang, H., Duan, W., Hu, J., Zhi, W., … Liu, J. (2016). GPER inhibits diabetes-mediated RhoA activation to prevent vascular endothelial dysfunction. *European Journal of Cell Biology*, *95*(2), 100–13. https://doi.org/10.1016/j.ejcb.2015.12.002

Lind, P. M., Örberg, J., Edlund, U.-B., Sjöblom, L., & Lind, L. (2004). The dioxin-like pollutant PCB 126 (3,3 ,4,4 ,5-pentachlorobiphenyl) affects risk factors for cardiovascular disease in female rats. *Toxicology Letters*, *150*, 293–299. https://doi.org/10.1016/j.toxlet.2004.02.008

Liu, S. V., Schally, A. V., Hawes, D., Xiong, S., Fazli, L., Gleave, M., … Pinski, J. (2010). Expression of Receptors for Luteinizing Hormone-Releasing Hormone (LH-RH) in Prostate Cancers following Therapy with LH-RH Agonists. *Clinical Cancer Research*, *16*(18), 4675–4680. https://doi.org/10.1158/1078-0432.CCR-10-1113

Liu, X., Tian, H., Li, H., Ge, C., Zhao, F., Yao, M., & Li, J. (2017). Derivate Isocorydine (d-ICD) Suppresses Migration and Invasion of Hepatocellular Carcinoma Cell by Downregulating ITGA1 Expression. *International Journal of Molecular Sciences*, *18*(3). https://doi.org/10.3390/ijms18030514

Liu, X., Yan, F., Yao, H., Chang, M., Qin, J., Li, Y., … Pei, X. (2014). Involvement of RhoA/ROCK in insulin secretion of pancreatic β-cells in 3D culture. *Cell and Tissue Research*, *358*(2), 359–369. https://doi.org/10.1007/s00441-014-1961-2

Lobo, T. F., Torloni, M. R., Mattar, R., Nakamura, M. U., Alexandre, S. M., & Daher, S. (2018). Adipokine levels in overweight women with early-onset gestational diabetes mellitus. *Journal of Endocrinological Investigation*. https://doi.org/10.1007/s40618-018-0894-0

LoPiccolo, J., Blumenthal, G. M., Bernstein, W. B., & Dennis, P. A. (2008). Targeting the PI3K/Akt/mTOR pathway: effective combinations and clinical considerations. *Drug Resistance Updates : Reviews and Commentaries in Antimicrobial and Anticancer Chemotherapy*, *11*(1–2), 32–50. https://doi.org/10.1016/j.drup.2007.11.003

Lou, Y.-R., Kanninen, L., Kuisma, T., Niklander, J., Noon, L. A., Burks, D., … Yliperttula, M. (2014). The use of nanofibrillar cellulose hydrogel as a flexible three-dimensional model to culture human pluripotent stem cells. *Stem Cells and Development*, *23*(4), 380–92. https://doi.org/10.1089/scd.2013.0314

Lunghi, L., Ferretti, M. E., Medici, S., Biondi, C., & Vesce, F. (2007). Control of human trophoblast function. *Reproductive Biology and Endocrinology*, *5*(1), 6. https://doi.org/10.1186/1477-7827-5-6

Machtinger, R., & Orvieto, R. (2014). Bisphenol A, oocyte maturation, implantation, and IVF outcome: Review of animal and human data. *Reproductive BioMedicine Online*, *29*(4), 404–410. https://doi.org/10.1016/j.rbmo.2014.06.013

Machtinger, R., Zhong, J., Mansur, A., Adir, M., Racowsky, C., Hauser, R., … Baccarelli, A. A. (2018). Placental lncRNA Expression Is Associated With Prenatal Phthalate Exposure. *Toxicological Sciences*. https://doi.org/10.1093/toxsci/kfy013

Mahalingam, S., Ther, L., Gao, L., Wang, W., Ziv-Gal, A., & Flaws, J. A. (2017). The effects of in utero bisphenol A exposure on ovarian follicle numbers and steroidogenesis in the F1 and F2 generations of mice. *Reproductive Toxicology*, *74*, 150–157. https://doi.org/10.1016/j.reprotox.2017.09.013

Malinen, M. M., Kanninen, L. K., Corlu, A., Isoniemi, H. M., Lou, Y.-R., Yliperttula, M. L., & Urtti, A. O. (2014). Differentiation of liver progenitor cell line to functional organotypic cultures in 3D nanofibrillar cellulose and hyaluronan-gelatin hydrogels. *Biomaterials*, *35*(19), 5110–5121. https://doi.org/10.1016/j.biomaterials.2014.03.020

Mallozzi, M., Bordi, G., Garo, C., & Caserta, D. (2016). The effect of maternal exposure to endocrine disrupting chemicals on fetal and neonatal development: A review on the major concerns. *Birth Defects Research Part C: Embryo Today: Reviews*, *108*(3), 224–242. https://doi.org/10.1002/bdrc.21137

Manikkam, M., Tracey, R., Guerrero-Bosagna, C., & Skinner, M. K. (2013). Plastics Derived Endocrine Disruptors (BPA, DEHP and DBP) Induce Epigenetic Transgenerational Inheritance of Obesity, Reproductive Disease and Sperm Epimutations. *PLoS ONE*, *8*(1), e55387. https://doi.org/10.1371/journal.pone.0055387

Mansouri, A., Cregut, M., Abbes, C., Durand, M.-J., Landoulsi, A., & Thouand, G. (2017). The Environmental Issues of DDT Pollution and Bioremediation: a Multidisciplinary Review. *Applied Biochemistry and Biotechnology*, *181*(1), 309–339. https://doi.org/10.1007/s12010-016-2214-5

Marchese, S., & Silva, E. (2012). Disruption of 3D MCF-12A breast cell cultures by estrogens--an in vitro model for ER-mediated changes indicative of hormonal carcinogenesis. *PloS One*, *7*(10), e45767. https://doi.org/10.1371/journal.pone.0045767

Marczell, I., Balogh, P., Nyiro, G., Kiss, A. L., Kovacs, B., Bekesi, G., … Patocs, A. (2018). Membrane-bound estrogen receptor alpha initiated signaling is dynamin dependent in breast cancer cells. *European Journal of Medical Research*, *23*(1), 31. https://doi.org/10.1186/s40001-018-0328-7

Marino, M., Ascenzi, P., & Acconcia, F. (2006). S-palmitoylation modulates estrogen receptor α localization and functions. *Steroids*, *71*(4), 298–303. https://doi.org/10.1016/j.steroids.2005.09.011

Marks, K. J., Hartman, T. J., Taylor, E. V, Rybak, M. E., Northstone, K., & Marcus, M. (2017). Exposure to phytoestrogens in utero and age at menarche in a contemporary British cohort. *Environmental Research*, *155*, 287–293. https://doi.org/10.1016/j.envres.2017.02.030

Masuzaki, H., Ogawa, Y., Sagawa, N., Hosoda, K., Matsumoto, T., Mise, H., … Nakao, K. (1997). Nonadipose tissue production of leptin: leptin as a novel placenta-derived hormone in humans. *Nature Medicine*, *3*(9), 1029–33. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9288733

McConkey, C. A., Delorme-Axford, E., Nickerson, C. A., Kim, K. S., Sadovsky, Y., Boyle, J. P., & Coyne, C. B. (2016). A three-dimensional culture system recapitulates placental syncytiotrophoblast development and microbial resistance. *Science Advances*, *2*(3), e1501462. https://doi.org/10.1126/sciadv.1501462

Meitzen, J., Luoma, J. I., Boulware, M. I., Hedges, V. L., Peterson, B. M., Tuomela, K., … Mermelstein, P. G. (2013). Palmitoylation of estrogen receptors is essential for neuronal membrane signaling. *Endocrinology*, *154*(11), 4293–304. https://doi.org/10.1210/en.2013-1172

Mennitti, L. V., Oliveira, J. L., Morais, C. A., Estadella, D., Oyama, L. M., Oller do Nascimento, C. M., & Pisani, L. P. (2015). Type of fatty acids in maternal diets during pregnancy and/or lactation and metabolic consequences of the offspring. *The Journal of Nutritional Biochemistry*, *26*(2), 99–111. https://doi.org/10.1016/j.jnutbio.2014.10.001

Messaros, B. M., Rossano, M. G., Liu, G., Diamond, M. P., Friderici, K., Nummy-

Jernigan, K., … Wirth, J. J. (2009). Negative effects of serum p,p 0 -DDE on sperm parameters and modification by genetic polymorphisms. https://doi.org/10.1016/j.envres.2009.02.009

Michałowicz, J. (2014). Bisphenol A - Sources, toxicity and biotransformation. *Environmental Toxicology and Pharmacology*, *37*(2), 738–758. https://doi.org/10.1016/j.etap.2014.02.003

Migda, M., Migda, M. S., Migda, B., & Wender-Ozegowska, E. (2017). Maternal first trimester parameters in the prediction of excessive fetal growth in pregnant women with metabolic syndrome. *Journal of Physiology and Pharmacology : An Official Journal of the Polish Physiological Society*, *68*(6), 833–839. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/29550795

Miodovnik, A., Engel, S. M., Zhu, C., Ye, X., Soorya, L. V, Silva, M. J., … Wolff, M. S. (2010). Endocrine disruptors and childhood social impairment. *Neurotoxicology*, *32*, 261–267. https://doi.org/10.1016/j.neuro.2010.12.009

Miossec, P., & Kolls, J. K. (2012). Targeting IL-17 and TH17 cells in chronic inflammation. *Nature Reviews Drug Discovery*, *11*(10), 763–776. https://doi.org/10.1038/nrd3794

Moe-Behrens, G. H. G., Klinger, F. G., Eskild, W., Grotmol, T., Haugen, T. B., & De Felici, M. (2003). Akt/PTEN Signaling Mediates Estrogen-Dependent Proliferation of Primordial Germ Cells *in Vitro*. *Molecular Endocrinology*, *17*(12), 2630–2638. https://doi.org/10.1210/me.2003-0006

Moers, A., Nieswandt, B., Massberg, S., Wettschureck, N., Grüner, S., Konrad, I., … Offermanns, S. (2003). G13 is an essential mediator of platelet activation in hemostasis and thrombosis. *Nature Medicine*, *9*(11), 1418–1422. https://doi.org/10.1038/nm943

Montes-Grajales, D., Fennix-Agudelo, M., & Miranda-Castro, W. (2017). Occurrence of personal care products as emerging chemicals of concern in water resources: A review. *Science of The Total Environment*, *595*, 601–614. https://doi.org/10.1016/j.scitotenv.2017.03.286

Morrissey, R. E., George, J. D., Price, C. J., Tyl, R. W., Marr, M. C., & KlMMELf, C. A. (1987). The Developmental Toxicity of Bisphenol A in Rats and Mice. *FUNDAMENTAL AND APPLIED TOXICOLOGY*, *8*(57), 1–582. Retrieved from https://watermark.silverchair.com/8-4-571.pdf?token=AQECAHi208BE49Ooan9kkhW_Ercy7Dm3ZL_9Cf3qfKAc485ysgAAAcEwggG9BgkqhkiG9w0BBwagggGuMIIBqgIBADCCAaMGCSqGSIb3DQEHATAeBglghkgBZQMEAS4wEQQMHxX8QMeQiSqjtKyoAgEQgIIBdDtH5H2X3O8uAuMWl0fV2CcRMyRgW4nm4feArni81NoSdTjOsyq_b5mhafhodg_mf_h5nyBM1-4VqP3k9m5ilJneL1FtXGDX3vkcLLlbiwykR0mxi-ac8CLm9B07crMP0cKHvXLkRgltRrSiN-U0EEPAx2xfV2WBHm4mIJ5d7UAXux30DhUClW_j3pnzsQvnGanQrfK51O-sUNqobmWPuyhS9AkhHcPa1AwthKAQd8DBAe5Bi4o-76JdXpc42u_aNy5rLz6V4OIlDy8JPXODhDvynRqJe5NkIaK9J0nLhZ3uk7WKozZKXB9S12k99X6vqA-aSB-NFZo_-gmrpjciuV14_keGabneBYyoK-ZMzqMXULJPiC6cEjHPR6qgrTcwCDOfZDIhCk0WzzeO9PdoYcDyC2cyQEkTk

Vfh0ySbC-LscxJ5BQI_q3a59NX54nMNLfqWyh8_RTL8MO5-2kRTjJ_v3NdwJuBUUQutCqjbjAYOK95Pwg

Mostafazadeh, M., Haiaty, S., Rastqar, A., & Keshvari, M. (2018). Correlation Between Resistin Level and Metabolic Syndrome Component: A Review. *Hormone and Metabolic Research*, *50*(07), 521–536. https://doi.org/10.1055/a-0637-1975

Motta-Mejia, C., Kandzija, N., Zhang, W., Mhlomi, V., Cerdeira, A. S., Burdujan, A., … Vatish, M. (2017). Placental Vesicles Carry Active Endothelial Nitric Oxide Synthase and Their Activity is Reduced in PreeclampsiaNovelty and Significance. *Hypertension*, *70*(2), 372–381. https://doi.org/10.1161/HYPERTENSIONAHA.117.09321

Muhamad, M. S., Salim, M. R., Lau, W. J., & Yusop, Z. (2016). A review on bisphenol A occurrences, health effects and treatment process via membrane technology for drinking water. *Environmental Science and Pollution Research*, *23*(12), 11549–11567. https://doi.org/10.1007/s11356-016-6357-2

Multhaup, A., Huppertz, B., Göhner, C., Böhringer, M., Mai, M., Markert, U., … Groten, T. (2018). N-cadherin knockdown leads to disruption of trophoblastic and endothelial cell interaction in a 3D cell culture model – New insights in trophoblast invasion failure. *Cell Adhesion & Migration*, *12*(3), 259–270. https://doi.org/10.1080/19336918.2017.1386822

Muoth, C., Wichser, A., Monopoli, M., Correia, M., Ehrlich, N., Loeschner, K., … Buerki-Thurnherr, T. (2016). A 3D co-culture microtissue model of the human placenta for nanotoxicity assessment. *Nanoscale*, *8*(39), 17322–17332. https://doi.org/10.1039/C6NR06749B

Murray, J., Eskenazi, B., Bornman, R., Gaspar, F. W., Crause, M., Obida, M., & Chevrier, J. (2018). Exposure to DDT and hypertensive disorders of pregnancy among South African women from an indoor residual spraying region: The VHEMBE study. *Environmental Research*, *162*, 49–54. https://doi.org/10.1016/j.envres.2017.12.006

Musicki, B., Pepe, G. J., & Albrecht, E. D. (2003). Functional differentiation of the placental syncytiotrophoblast: Effect of estrogen on chorionic somatomammotropin expression during early primate pregnancy. *The Journal of Clinical Endocrinology and Metabolism*, *88*(9), 4316–23. https://doi.org/10.1210/jc.2002-022052

Nadal, A., Ropero, A. B., Laribi, O., Maillet, M., Fuentes, E., & Soria, B. (2000). Nongenomic actions of estrogens and xenoestrogens by binding at a plasma membrane receptor unrelated to estrogen receptor alpha and estrogen receptor beta. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(21), 11603–8. https://doi.org/10.1073/pnas.97.21.11603

Nagel, S. C., Hagelbarger, J. L., & McDonnell, D. P. (2001). Development of an ER Action Indicator Mouse for the Study of Estrogens, Selective ER Modulators (SERMs), and Xenobiotics. *Endocrinology*, *142*(11), 4721–4728. https://doi.org/10.1210/endo.142.11.8471

Nephew, K. P., Long, X., Osborne, E., Burke, K. A., Ahluwalia, A., & Bigsby, R. M.

(2000). Effect of estradiol on estrogen receptor expression in rat uterine cell types. *Biol Reprod*, *62*(1), 168–177. https://doi.org/10.1095/biolreprod62.1.168

Newbold, R. R., Padilla-Banks, E., & Jefferson, W. N. (2009). Environmental estrogens and obesity. *Molecular and Cellular Endocrinology*, *304*(1–2), 84–9. https://doi.org/10.1016/j.mce.2009.02.024

Ngala, R. A., Fondjo, L. A., Gmagna, P., Ghartey, F. N., & Awe, M. A. (2017). Placental peptides metabolism and maternal factors as predictors of risk of gestational diabetes in pregnant women. A case-control study. *PloS One*, *12*(7), e0181613. https://doi.org/10.1371/journal.pone.0181613

Niikawa, H., Suzuki, T., Miki, Y., Suzuki, S., Nagasaki, S., Akahira, J., … Sasano, H. (2008). Intratumoral estrogens and estrogen receptors in human non-small cell lung carcinoma. *Clinical Cancer Research*, *14*(14), 4417–4426. https://doi.org/10.1158/1078-0432.CCR-07-1950

Nobes, C. D., Hawkins, P., Stephens, L., & Hall, A. (1995). Activation of the small GTP-binding proteins rho and rac by growth factor receptors. *Journal of Cell Science*, *108*(1).

O'Tierney-Ginn, P. F., & Lash, G. E. (2014). Beyond pregnancy: Modulation of trophoblast invasion and its consequences for fetal growth and long-term children's health. *Journal of Reproductive Immunology*, *104–105*(1), 37–42. https://doi.org/10.1016/j.jri.2014.04.002

Oesterreich, S., Zhang, P., Guler, R. L., Sun, X., Curran, E. M., Welshons, W. V, … Lee, A. V. (2001). Re-expression of Estrogen Receptor ␣ in Estrogen Receptor ␣-negative MCF-7 Cells Restores both Estrogen and Insulin-like Growth Factor-mediated Signaling and Growth. *CANCER RESEARCH*, *61*, 5771–5777. Retrieved from http://cancerres.aacrjournals.org/content/canres/61/15/5771.full.pdf

Olival Costa, H., de Castro Neto, N. P., Mara Rossi, L., Millas, I., Coelho, F., & da Silva, L. (2014). Influence of estradiol administration on estrogen receptors of nasal mucosa: an experimental study on guinea pigs☆☆Please cite this article as: Costa HO, Castro-Neto NP, Rossi LM, Millas I, Coelho F, Silva L. Influence of estradiol administration on estro. *Brazilian Journal of Otorhinolaryngology*, *80*(1), 18–23. https://doi.org/10.5935/1808-8694.20140006

Olson, M. F., Ashworth, A., & Hall, A. (1995). An essential role for Rho, Rac, and Cdc42 GTPases in cell cycle progression through G1. *Science (New York, N.Y.)*, *269*(5228), 1270–2. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7652575

Olson, M. F., Paterson, H. F., & Marshall, C. J. (1998). Signals from Ras and Rho GTPases interact to regulate expression of p21Waf1/Cip1. *Nature*, *394*(6690), 295–299. https://doi.org/10.1038/28425

Orendi, K., Gauster, M., Moser, G., Meiri, H., & Huppertz, B. (2010). The choriocarcinoma cell line BeWo: Syncytial fusion and expression of syncytium-specific proteins. *Reproduction*, *140*(5), 759–766. https://doi.org/10.1530/REP-

10-0221

Orendi, K., Kivity, V., Sammar, M., Grimpel, Y., Gonen, R., Meiri, H., … Huppertz, B. (2011). Placental and trophoblastic in vitro models to study preventive and therapeutic agents for preeclampsia. *Placenta*, *32*(SUPPL. 1), S49–S54. https://doi.org/10.1016/j.placenta.2010.11.023

Owman, C., Blay, P., Nilsson, C., & Lolait, S. J. (1996). Cloning of human cDNA encoding a novel heptahelix receptor expressed in Burkitt's lymphoma and widely distributed in brain and peripheral tissues. *Biochemical and Biophysical Research Communications*, *228*(2), 285–292. https://doi.org/10.1006/bbrc.1996.1654

Palanza, P., Nagel, S. C., Parmigiani, S., & Vom Saal, F. S. (2016). Perinatal exposure to endocrine disruptors: sex, timing and behavioral endpoints. *Current Opinion in Behavioral Sciences*, *7*, 69–75. https://doi.org/10.1016/j.cobeha.2015.11.017

Pan, X., Wang, X., Wang, Z., Wang, X., Dou, Z., & Li, Z. (2015). Bisphenol a influences blastocyst implantation via regulating integrin ??3 and trophinin expression levels. *International Journal of Clinical and Experimental Medicine*, *8*(11), 20035–20045.

Pan, Y., Zhou, F., He, C., Hui, L., Huang, T., & Wei, Y. (2017). Leptin-LepRb Expressed in Gastric Cancer Patients and Related to Cancer-Related Depression. *BioMed Research International*, *2017*, 6482842. https://doi.org/10.1155/2017/6482842

Park, C., Choi, W., Hwang, M., Lee, Y., Kim, S., Yu, S., … Choi, K. (2017). Associations between urinary phthalate metabolites and bisphenol A levels, and serum thyroid hormones among the Korean adult population - Korean National Environmental Health Survey (KoNEHS) 2012-2014. *Science of the Total Environment*, *584–585*, 950–957. https://doi.org/10.1016/j.scitotenv.2017.01.144

Park, C. J., & Gye, M. C. (2014). Sensitization of vitellogenin gene expression by low doses of octylphenol is mediated by estrogen receptor autoregulation in the Bombina orientalis (Boulenger) male liver. *Aquatic Toxicology*, *156*, 191–200. https://doi.org/10.1016/j.aquatox.2014.08.013

Patisaul, H. B., & Jefferson, W. (2010). The pros and cons of phytoestrogens. *Frontiers in Neuroendocrinology*, *31*(4), 400–19. https://doi.org/10.1016/j.yfrne.2010.03.003

Pattillo, R. A., & Gey, G. O. (1968). The Establishment Trophoblastic of a Cell Line of Human Cells in Vitro1, (July), 1231–1236.

Paulose, T., Speroni, L., Sonnenschein, C., & Soto, A. M. (2015). Estrogens in the wrong place at the wrong time: Fetal BPA exposure and mammary cancer. *Reproductive Toxicology*, *54*, 58–65. https://doi.org/10.1016/j.reprotox.2014.09.012

Pawoo, N., & Heller, D. S. (2014). Placental Mesenchymal Dysplasia. *Archives of Pathology & Laboratory Medicine*, *138*(9), 1247–1249. https://doi.org/10.5858/arpa.2013-0399-RS

Peretz, J., Gupta, R. K., Singh, J., Hernández-Ochoa, I., & Flaws, J. A. (2011).

Bisphenol A Impairs Follicle Growth, Inhibits Steroidogenesis, and Downregulates Rate-Limiting Enzymes in the Estradiol Biosynthesis Pathway. *Toxicological Sciences*, *119*(1), 209–217. https://doi.org/10.1093/toxsci/kfq319

Peretz, J., Vrooman, L., Ricke, W. A., Hunt, P. A., Ehrlich, S., Hauser, R., … Flaws, J. A. (2014). Bisphenol A and reproductive health: Update of experimental and human evidence, 2007-2013. *Environmental Health Perspectives*, *122*(8), 775–786. https://doi.org/10.1289/ehp.1307728

Perez-Moreno, M., Jamora, C., & Fuchs, E. (2003). Sticky Business: Orchestrating Cellular Signals at Adherens Junctions. *Cell*, *112*(4), 535–548. https://doi.org/10.1016/S0092-8674(03)00108-9

Peters, J. L., Fabian, M. P., & Levy, J. I. (2014). Combined impact of lead, cadmium, polychlorinated biphenyls and non-chemical risk factors on blood pressure in NHANES. https://doi.org/10.1016/j.envres.2014.03.038

Petry, C. J. (2018). Gestational diabetes: risk factors and recent advances in its genetics and treatment. https://doi.org/10.1017/S0007114510001741

Piccart, M., Parker, L. M., & Pritchard, K. I. (2003). Oestrogen receptor downregulation: an opportunity for extending the window of endocrine therapy in advanced breast cancer. *Annals of Oncology : Official Journal of the European Society for Medical Oncology*, *14*(7), 1017–25. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12853342

Pickl, M., & Ries, C. H. (2009). Comparison of 3D and 2D tumor models reveals enhanced HER2 activation in 3D associated with an increased response to trastuzumab. *Oncogene*, *28*(3), 461–468. https://doi.org/10.1038/onc.2008.394

Pirsaheb, M., Limoee, M., Namdari, F., & Khamutian, R. (2015). Organochlorine pesticides residue in breast milk: a systematic review. *Medical Journal of the Islamic Republic of Iran (MJIRI)*. Retrieved from http://mjiri.iums.ac.ir

Plummer, P. N., Colson, N. J., Lewohl, J. M., Mackay, R. K., Fernandez, F., Haupt, L. M., & Griffiths, L. R. (2011). Significant differences in gene expression of GABA receptors in peripheral blood leukocytes of migraineurs. *Gene*, *490*, 32–36. https://doi.org/10.1016/j.gene.2011.08.031

Pongcharoen, S., Somran, J., Sritippayawan, S., Niumsup, P., Chanchan, P., Butkhamchot, P., … Searle, R. F. (2007). Interleukin-17 Expression in the Human Placenta. *Placenta*, *28*(1), 59–63. https://doi.org/10.1016/j.placenta.2006.01.016

Popova, P., Vasilyeva, L., Tkachuck, A., Puzanov, M., Golovkin, A., Bolotko, Y., … Grineva, E. (2018). A Randomised, Controlled Study of Different Glycaemic Targets during Gestational Diabetes Treatment: Effect on the Level of Adipokines in Cord Blood and ANGPTL4 Expression in Human Umbilical Vein Endothelial Cells. *International Journal of Endocrinology*, *2018*, 1–8. https://doi.org/10.1155/2018/6481658

Portman, M. A., Navarro, S. L., Bruce, M. E., & Lampe, J. W. (2016). Soy isoflavone intake is associated with risk of Kawasaki disease. *Nutrition Research (New York, N.Y.)*, *36*(8), 827–34. https://doi.org/10.1016/j.nutres.2016.04.002

Post, G. R., Collins, L. R., Kennedy, E. D., Moskowitz, S. A., Aragay, A. M., Goldstein, D., … Bourne, H. R. (1996). Coupling of the Thrombin Receptor to G12 May Account for Selective Effects of Thrombin on Gene Expression and DNA Synthesis in 1321N1 Astrocytoma Cells. *Molecular Biology of the Cell*, *7*, 1679–1690. Retrieved from https://www.molbiolcell.org/doi/pdf/10.1091/mbc.7.11.1679

Pottenger, L. H., Domoradzki, J. Y., Markham, D. a, Hansen, S. C., Cagen, S. Z., & Waechter, J. M. (2000). The relative bioavailability and metabolism of bisphenol A in rats is dependent upon the route of administration. *Toxicological Sciences : An Official Journal of the Society of Toxicology*, *54*(1), 3–18. https://doi.org/10.1093/toxsci/54.1.3

Prossnitz, E. R., & Arterburn, J. B. (2015). International Union of Basic and Clinical Pharmacology. XCVII. G Protein-Coupled Estrogen Receptor and Its Pharmacologic Modulators. *Pharmacological Reviews*, *67*(3), 505–540. https://doi.org/10.1124/pr.114.009712

Pruitt, D. L., Meserve, L. A., & Bingman, V. P. (1999). Reduced growth of intra-and infra-pyramidal mossy fibers is produced by continuous exposure to polychlorinated biphenyl. *Toxicology*, *138*, 11–17. Retrieved from www.elsevier.com/locate/toxicol

Prusinski, L., Al-Hendy, A., & Yang, Q. (2016). Developmental exposure to endocrine disrupting chemicals alters the epigenome: Identification of reprogrammed targets. *Gynecology and Obstetrics Research : Open Journal*, *3*(1), 1–6. https://doi.org/10.17140/GOROJ-3-127

Quinete, N., Esser, A., Kraus, T., & Schettgen, T. (2017). PCB 28 metabolites elimination kinetics in human plasma on a real case scenario: Study of hydroxylated polychlorinated biphenyl (OH-PCB) metabolites of PCB 28 in a highly exposed German Cohort. *Toxicology Letters*, *276*, 100–107. https://doi.org/10.1016/j.toxlet.2017.05.025

Raghupathy, R., Al Mutawa, E., Makhseed, M., Azizieh, F., & Szekeres-Bartho, J. (2005). Modulation of cytokine production by dydrogesterone in lymphocytes from women with recurrent miscarriage. *BJOG: An International Journal of Obstetrics & Gynaecology*, *112*(8), 1096–1101. https://doi.org/10.1111/j.1471-0528.2005.00633.x

Rahman, M. S., Kwon, W. S., Lee, J. S., Yoon, S. J., Ryu, B. Y., & Pang, M. G. (2015). Bisphenol-A Affects Male Fertility via Fertility-related Proteins in Spermatozoa. *Scientific Reports*, *5*(1), 1–9. https://doi.org/10.1038/srep09169

Ranscht, B. (1994). Cadherins and catenins: interactions and functions in embryonic development. *Current Opinion in Cell Biology*, *6*(5), 740–746. https://doi.org/10.1016/0955-0674(94)90102-3

Rebut-Bonneton, C., Boutemy-Roulier, S., & Evain-Brion, D. (1993). Modulation of pp60c-src activity and cellular localization during differentiation of human trophoblast cells in culture. *Journal of Cell Science*, *105 (Pt 3)*, 629–36. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7691846

Red-Horse, K., Kapidzic, M., Zhou, Y., Feng, K.-T., Singh, H., Fisher, S. J., &

Weinstein, B. M. (2005). EPHB4 regulates chemokine-evoked trophoblast responses: a mechanism for incorporating the human placenta into the maternal circulation. *Development (Cambridge, England)*, *132*(18), 4097–106. https://doi.org/10.1242/dev.01971

Reddivari, L., Veeramachaneni, D. N. R., Walters, W. A., Lozupone, C., Palmer, J., Hewage, M. K. K., … Vanamala, J. K. P. (2017). Perinatal Bisphenol A Exposure Induces Chronic Inflammation in Rabbit Offspring via Modulation of Gut Bacteria and Their Metabolites. *MSystems*, *2*(5). https://doi.org/10.1128/mSystems.00093-17

Reed, C. E., & Fenton, S. E. (2013). Exposure to diethylstilbestrol during sensitive life stages: a legacy of heritable health effects. *Birth Defects Research. Part C, Embryo Today : Reviews*, *99*(2), 134–46. https://doi.org/10.1002/bdrc.21035

Reger, M. K., Zollinger, T. W., Liu, Z., Jones, J., & Zhang, J. (2016). Urinary phytoestrogens and cancer, cardiovascular, and all-cause mortality in the continuous National Health and Nutrition Examination Survey. *European Journal of Nutrition*, *55*(3), 1029–1040. https://doi.org/10.1007/s00394-015-0917-y

Resnik, R., Killam, A. P., Battaglia, F. C., Makowski, E. L., & Meschia, G. (1974). The Stimulation of Uterine Blood Flow by Various Estrogens 1. *Endocrinology*, *94*(4), 1192–1196. https://doi.org/10.1210/endo-94-4-1192

Revankar, C. M., Cimino, D. F., Sklar, L. A., Arterburn, J. B., & Prossnitz, E. R. (2005). A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science (New York, N.Y.)*, *307*(5715), 1625–30. https://doi.org/10.1126/science.1106943

Ribeiro, E., Ladeira, C., & Viegas, S. (2017). EDCs Mixtures: A Stealthy Hazard for Human Health? *Toxics*, *5*(1). https://doi.org/10.3390/toxics5010005

Richter, C. A., Birnbaum, L. S., Farabollini, F., Newbold, R. R., Rubin, B. S., Talsness, C. E., … vom Saal, F. S. (2007). In vivo effects of bisphenol A in laboratory rodent studies. *Reproductive Toxicology*, *24*(2), 199–224. https://doi.org/10.1016/j.reprotox.2007.06.004

Riedl, A., Schlederer, M., Pudelko, K., Stadler, M., Walter, S., Unterleuthner, D., … Dolznig, H. (2017). Comparison of cancer cells in 2D vs 3D culture reveals differences in AKT–mTOR–S6K signaling and drug responses. *Journal of Cell Science*, *130*(1), 203–218. https://doi.org/10.1242/jcs.188102

Riva, E., Grandi, F., Massetto, N., Radaelli, G., Giovannini, M., Zetterström, R., & Agostoni, C. (2004). Poly chlorinated biphenyls in colostral milk and visual function at 12 months of life. *Acta Paediatrica*, *93*(8), 1103–1107. https://doi.org/10.1111/j.1651-2227.2004.tb02724.x

Robertson, J. A., Farnell, Y., Lindahl, L. S., & Ing, N. H. (2002). Estradiol up-regulates estrogen receptor messenger ribonucleic acid in endometrial carcinoma (Ishikawa) cells by stabilizing the message. *Journal of Molecular Endocrinology*, *29*(1), 125–35. https://doi.org/10.1677/JME.0.0290125

Robins, J. C., Marsit, C. J., Padbury, J. F., & Sharma, S. S. (2011). Endocrine

disruptors, environmental oxygen, epigenetics and pregnancy. *Frontiers in Bioscience (Elite Edition)*, *3*, 690–700. https://doi.org/10.1016/j.bbi.2008.05.010

Rosenbaum, P. F., Weinstock, R. S., Silverstone, A. E., Sjödin, A., & Pavuk, M. (2017). Metabolic syndrome is associated with exposure to organochlorine pesticides in Anniston, AL, United States. *Environment International*, *108*, 11–21. https://doi.org/10.1016/j.envint.2017.07.017

Rubin, B. S., Murray, M. K., Damassa, D. A., King, J. C., & Soto, A. M. (2001). Perinatal exposure to low doses of bisphenol A affects body weight, patterns of estrous cyclicity, and plasma LH levels. *Environmental Health Perspectives*, *109*(7), 675–680. https://doi.org/10.1289/ehp.01109675

Saceda, M., Lippman, M. E., Chambon, P., Lindsey, R. L., Ponglikitmongkol, M., Puente, M., & Martin, M. B. (1988). Regulation of the estrogen receptor in MCF-7 cells by estradiol. *Molecular Endocrinology (Baltimore, Md.)*, *2*(12), 1157–62. https://doi.org/10.1210/mend-2-12-1157

Sandquist, I., & Kolls, J. (2018). Update on regulation and effector functions of Th17 cells. *F1000Research*, *7*, 205. https://doi.org/10.12688/f1000research.13020.1

Santamaría, C., Durando, M., Muñoz De Toro, M., Luque, E. H., & Rodriguez, H. A. (2016). Ovarian dysfunctions in adult female rat offspring born to mothers perinatally exposed to low doses of bisphenol A. https://doi.org/10.1016/j.jsbmb.2015.11.016

Sato, B. L., Ward, M. A., Astern, J. M., Kendal-Wright, C. E., & Collier, A. C. (2015). Validation of murine and human placental explant cultures for use in sex steroid and phase II conjugation toxicology studies. *Toxicology in Vitro : An International Journal Published in Association with BIBRA*, *29*(1), 103–12. https://doi.org/10.1016/j.tiv.2014.09.008

Schecter, A., Cramer, P., Boggess, K., Stanley, J., Päpke, O., Olson, J., … Schmitz, M. (2001). Intake Of Dioxins and Related Compounds from Food in the U.S. Population. *Journal of Toxicology and Environmental Health, Part A*, *63*(1), 1–18. https://doi.org/10.1080/152873901750128326

Schiessl, B., Mylonas, I., Kuhn, C., Kunze, S., Schulze, S., Friese, K., & Jeschke, U. (2006). Expression of Estrogen Receptor-α, Estrogen Receptor-β and Placental Endothelial and Inducible NO Synthase in Intrauterine Growth-Restricted and Normal Placentals. *Archives of Medical Research*, *37*(8), 967–975. https://doi.org/10.1016/j.arcmed.2006.03.011

Schmidt, M. I., Duncan, B. B., Reichelt, A. J., Branchtein, L., Matos, M. C., Forti, A. C. e, … Yamashita, T. (2001). Gestational Diabetes Mellitus Diagnosed With a 2-h 75-g Oral Glucose Tolerance Test and Adverse Pregnancy Outcomes. *Diabetes Care*, *24*(7), 1151–1155. https://doi.org/10.2337/DIACARE.24.7.1151

Schönfelder, G., Wittfoht, W., Hopp, H., Talsness, C. E., Paul, M., & Chahoud, I. (2002). Parent bisphenol A accumulation in the human maternal-fetal-placental unit. *Environmental Health Perspectives*, *110*(11), A703-7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12417499

Schwartz, D. R., & Lazar, M. A. (2011). Human resistin: found in translation from mouse to man. *Trends in Endocrinology and Metabolism: TEM*, *22*(7), 259–65. https://doi.org/10.1016/j.tem.2011.03.005

Schwartz, M. (2004). Rho signalling at a glance. *Journal of Cell Science*, *117*(Pt 23), 5457–8. https://doi.org/10.1242/jcs.01582

Seeho, S. K. M., Park, J. H., Rowe, J., Morris, J. M., & Gallery, E. D. M. (2008). Villous explant culture using early gestation tissue from ongoing pregnancies with known normal outcomes: The effect of oxygen on trophoblast outgrowth and migration. *Human Reproduction*, *23*(5), 1170–1179. https://doi.org/10.1093/humrep/den066

Seol, H.-J., Oh, M.-J., Yeo, M.-K., Kim, A., Lee, E.-S., & Kim, H.-J. (2010). Comparison of Serum Levels and the Placental Expression of Resistin Between Patients with Preeclampsia and Normal Pregnant Women. *Hypertension in Pregnancy*, *29*(3), 310–317. https://doi.org/10.3109/10641950902849850

Shang, M., Dong, X., & Hou, L. (2018). Correlation of adipokines and markers of oxidative stress in women with gestational diabetes mellitus and their newborns. *Journal of Obstetrics and Gynaecology Research*. https://doi.org/10.1111/jog.13586

Shen, K., Yu, W., Yu, Y., Liu, X., & Cui, X. (2018). Knockdown of TMEM45B inhibits cell proliferation and invasion in gastric cancer. *Biomedicine & Pharmacotherapy*, *104*, 576–581. https://doi.org/10.1016/j.biopha.2018.05.016

Sheng, Z.-G., Huang, W., Liu, Y.-X., & Zhu, B.-Z. (2013). Bisphenol A at a low concentration boosts mouse spermatogonial cell proliferation by inducing the G protein-coupled receptor 30 expression. *Toxicology and Applied Pharmacology*, *267*, 88–94. https://doi.org/10.1016/j.taap.2012.12.014

Shi, X.-Y., Wang, Z., Liu, L., Feng, L.-M., Li, N., Liu, S., & Gao, H. (2017). Low concentrations of bisphenol A promote human ovarian cancer cell proliferation and glycolysis-based metabolism through the estrogen receptor-α pathway. *Chemosphere*, *185*, 361–367. https://doi.org/10.1016/J.CHEMOSPHERE.2017.07.027

Sidarala, V., & Kowluru, A. (2017). The Regulatory Roles of Mitogen-Activated Protein Kinase (MAPK) Pathways in Health and Diabetes: Lessons Learned from the Pancreatic β-Cell. *Recent Patents on Endocrine, Metabolic & Immune Drug Discovery*, *10*(2), 76–84. https://doi.org/10.2174/1872214810666161020154905

Simmons, S. L., Cummings, J. A., Clemens, L. G., & Nunez, A. A. (2004). Exposure to PCB 77 affects the maternal behavior of rats. https://doi.org/10.1016/j.physbeh.2004.10.022

Skinner, M. K., Manikkam, M., & Guerrero-Bosagna, C. (2011). Epigenetic transgenerational actions of endocrine disruptors. *Reproductive Toxicology (Elmsford, N.Y.)*, *31*(3), 337–43. https://doi.org/10.1016/j.reprotox.2010.10.012

Spiegelman, B. M., & Flier, J. S. (2001). Obesity and the regulation of energy balance. *Cell*, *104*(4), 531–43. https://doi.org/10.1016/S0092-8674(01)00240-9

Srivastava, S., Gupta, P., Chandolia, A., & Alam, I. (2015). Bisphenol A : A Threat to Human Health ? *Journal of Environmental Health*, *77*(6), 20–26.

Steenland, K., Hein, M. J., Cassinelli, R. T., Prince, M. M., Nilsen, N. B., Whelan, E. A., … Schnorr, T. M. (2006). Polychlorinated Biphenyls and Neurodegenerative Disease Mortality in an Occupational Cohort. *Epidemiology*, *17*(1), 8–13. https://doi.org/10.1097/01.ede.0000190707.51536.2b

Steinberg, M., & Robins, J. (2016). Cellular Models of Trophoblast Differentiation. *Seminars in Reproductive Medicine*, *34*(01), 050–056. https://doi.org/10.1055/s-0035-1570026

Steinmetz, R., Mitchner, N. A., Grant, A., Allen, D. L., Bigsby, R. M., & Ben-Jonathan, N. (1998). The Xenoestrogen Bisphenol A Induces Growth, Differentiation, and c-*fos* Gene Expression in the Female Reproductive Tract [1]. *Endocrinology*, *139*(6), 2741–2747. https://doi.org/10.1210/endo.139.6.6027

Steppan, C. M., Bailey, S. T., Bhat, S., Brown, E. J., Banerjee, R. R., Wright, C. M., … Lazar, M. A. (2001). The hormone resistin links obesity to diabetes. *Nature*, *409*(6818), 307–312. https://doi.org/10.1038/35053000

Stulc, J. (1997). Placental transfer of inorganic ions and water. *Physiol Rev*, *77*(July), 805–836. https://doi.org/10.1152/physrev.1997.77.3.805

Sun, S.-C. (2011). Non-canonical NF-κB signaling pathway. *Cell Research*, *21*(1), 71–85. https://doi.org/10.1038/cr.2010.177

Swan, S. H., Liu, F., Hines, M., Kruse, R. L., Wang, C., Redmon, J. B., … Weiss, B. (2010). Prenatal phthalate exposure and reduced masculine play in boys. *International Journal of Andrology*, *33*(2), 259–269. https://doi.org/10.1111/j.1365-2605.2009.01019.x

Sweeting, A. N., Wong, J., Appelblom, H., Ross, G. P., Kouru, H., Williams, P. F., … Hyett, J. A. (2018). A Novel Early Pregnancy Risk Prediction Model for Gestational Diabetes Mellitus. *Fetal Diagnosis and Therapy*, 1–9. https://doi.org/10.1159/000486853

Takagi, A., Watanabe, M., Ishii, Y., Morita, J., Hirokawa, Y., Matsuzaki, T., & Shiraishi, T. (2007). Three-dimensional cellular spheroid formation provides human prostate tumor cells with tissue-like features. *Anticancer Research*, *27*(1 A), 45–54.

Takai, Y., Tsutsumi, O., Ikezuki, Y., Hiroi, H., Osuga, Y., Momoeda, M., … Taketani, Y. (2000). Estrogen receptor-mediated effects of a xenoestrogen, bisphenol A, on preimplantation mouse embryos. *Biochemical and Biophysical Research Communications*, *270*(3), 918–921. https://doi.org/10.1006/bbrc.2000.2548

Takenaka, N., Nihata, Y., Ueda, S., & Satoh, T. (2017). In situ detection of the activation of Rac1 and RalA small GTPases in mouse adipocytes by immunofluorescent microscopy following in vivo and ex vivo insulin stimulation. https://doi.org/10.1016/j.cellsig.2017.08.004

Takeuchi, T., Tsutsumi, O., Ikezuki, Y., Takai, Y., & Taketani, Y. (2004). Positive

Relationship between Androgen and the Endocrine Disruptor, Bisphenol A, in Normal Women and Women with Ovarian Dysfunction. *Endocrine Journal*, *51*(2), 165–169. https://doi.org/10.1507/endocrj.51.165

Tal, R., Taylor, H. S., Burney, R. O., Mooney, S. B., & Giudice, L. C. (2000). *Endocrinology of Pregnancy*. *Endotext*. MDText.com, Inc. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/25905197

Tao, W., Wu, J., Xie, B.-X., Zhao, Y.-Y., Shen, N., Jiang, S., … Li, C.-J. (2015). Lipid-induced Muscle Insulin Resistance Is Mediated by GGPPS via Modulation of the RhoA/Rho Kinase Signaling Pathway. *The Journal of Biological Chemistry*, *290*(33), 20086–97. https://doi.org/10.1074/jbc.M115.657742

Taylor, R. N., Newman, E. D., & Chen, S. A. (1991). Forskolin and methotrexate induce an intermediate trophoblast phenotype in cultured human choriocarcinoma cells. *American Journal of Obstetrics and Gynecology*, *164*(1 Pt 1), 204–10. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1986609

Teitelbaum, S. L., Mervish, N., Moshier, E. L., Vangeepuram, N., Galvez, M. P., Calafat, A. M., … Wolff, M. S. (2012). Associations between phthalate metabolite urinary concentrations and body size measures in New York City children. https://doi.org/10.1016/j.envres.2011.12.006

Téllez-Rojo, M. M., Cantoral, A., Cantonwine, D. E., Schnaas, L., Peterson, K., Hu, H., & Meeker, J. D. (2013). Prenatal urinary phthalate metabolites levels and neurodevelopment in children at two and three years of age. https://doi.org/10.1016/j.scitotenv.2013.05.021

Thayer, K. A., Doerge, D. R., Hunt, D., Schurman, S. H., Twaddle, N. C., Churchwell, M. I., … Birnbaum, L. S. (2015). Pharmacokinetics of bisphenol A in humans following a single oral administration. *Environment International*, *83*, 107–115. https://doi.org/10.1016/j.envint.2015.06.008

Tohmé, M., Prud'homme, S. M., Boulahtouf, A., Samarut, E., Brunet, F., Bernard, L., … Laudet, V. (2014). Estrogen-related receptor γ is an *in vivo* receptor of bisphenol A. *The FASEB Journal*, *28*(7), 3124–3133. https://doi.org/10.1096/fj.13-240465

Toichuev, R. M., Zhilova, L. V., Paizildaev, T. R., Khametova, M. S., Rakhmatillaev, A., Sakibaev, K. S., … Lichtensteiger, W. (2017). Organochlorine pesticides in placenta in Kyrgyzstan and the effect on pregnancy, childbirth, and newborn health. *Environmental Science and Pollution Research*, 1–10. https://doi.org/10.1007/s11356-017-0962-6

Tong, C., Feng, X., Chen, J., Qi, X., Zhou, L., Shi, S., … Zhang, H. (2016). G protein-coupled receptor 30 regulates trophoblast invasion and its deficiency is associated with preeclampsia. *Journal of Hypertension*, *34*(4), 710–8. https://doi.org/10.1097/HJH.0000000000000844

Toth, P., Li, X., Rao, C. V, Lincoln, S. R., Sanfilippo, J. S., Spinnato, J. A., & Yussman, M. A. (1994). Expression of functional human chorionic gonadotropin/human luteinizing hormone receptor gene in human uterine arteries. *The Journal of Clinical Endocrinology & Metabolism*, *79*(1), 307–315.

https://doi.org/10.1210/jcem.79.1.8027246

Trapphoff, T., Heiligentag, M., El Hajj, N., Haaf, T., & Eichenlaub-Ritter, U. (2013). Chronic exposure to a low concentration of bisphenol A during follicle culture affects the epigenetic status of germinal vesicles and metaphase II oocytes. *Fertility and Sterility*, *100*(6), 1758–1767.e1. https://doi.org/10.1016/j.fertnstert.2013.08.021

Trasande, L., Attina, T. M., Sathyanarayana, S., Spanier, A. J., & Blustein, J. (2013). Race/Ethnicity-Specific Associations of Urinary Phthalates with Childhood Body Mass in a Nationally Representative Sample. *Environmental Health Perspectives*, *121*(4), 501–506. https://doi.org/10.1289/ehp.1205526

Trasande, L., Spanier, A. J., Sathyanarayana, S., Attina, T. M., & Blustein, J. (2013). Urinary Phthalates and Increased Insulin Resistance in Adolescents. *PEDIATRICS*, *132*(3), e646–e655. https://doi.org/10.1542/peds.2012-4022

Trdan Lušin, T., Roškar, R., & Mrhar, A. (2012). Evaluation of bisphenol A glucuronidation according to UGT1A1*28 polymorphism by a new LC–MS/MS assay. *Toxicology*, *292*(1), 33–41. https://doi.org/10.1016/j.tox.2011.11.015

Tsiotra, P. C., Halvatsiotis, P., Patsouras, K., Maratou, E., Salamalekis, G., Raptis, S. A., … Boutati, E. (2018). Circulating adipokines and mRNA expression in adipose tissue and the placenta in women with gestational diabetes mellitus. https://doi.org/10.1016/j.peptides.2018.01.005

Tuckey, R. C. (2005). Progesterone synthesis by the human placenta. *Placenta*, *26*(4), 273–281. https://doi.org/10.1016/j.placenta.2004.06.012

Tunster, S. J., Van de Pette, M., Creeth, H. D. J., Lefebvre, L., & John, R. M. (2018). Fetal growth restriction in a genetic model of sporadic Beckwith-Wiedemann Syndrome. *Disease Models & Mechanisms*, dmm.035832. https://doi.org/10.1242/dmm.035832

Tyl, R. W. (2014). Abbreviated assessment of bisphenol A toxicology literature. *Seminars in Fetal and Neonatal Medicine*, *19*(3), 195–202. https://doi.org/10.1016/j.siny.2013.11.010

Ueda, S., Kataoka, T., & Satoh, T. (2008). Activation of the small GTPase Rac1 by a specific guanine-nucleotide-exchange factor suffices to induce glucose uptake into skeletal-muscle cells. *Biology of the Cell*, *100*(11), 645–661. https://doi.org/10.1042/BC20070160

Ueda, S., Kitazawa, S., Ishida, K., Nishikawa, Y., Matsui, M., Matsumoto, H., … Satoh, T. (2010). Crucial role of the small GTPase Rac1 in insulin-stimulated translocation of glucose transporter 4 to the mouse skeletal muscle sarcolemma. *The FASEB Journal*, *24*(7), 2254–2261. https://doi.org/10.1096/fj.09-137380

Vandenberg, L. N., Chahoud, I., Heindel, J. J., Padmanabhan, V., Paumgartten, F. J. R., & Schoenfelder, G. (2010). Urinary, circulating, and tissue biomonitoring studies indicate widespread exposure to bisphenol A. *Environmental Health Perspectives*, *118*(8), 1055–70. https://doi.org/10.1289/ehp.0901716

Vandenberg, L. N., Colborn, T., Hayes, T. B., Heindel, J. J., Jacobs, D. R., Lee, D.-H., … Myers, J. P. (2012). Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. *Endocrine Reviews*, *33*(3), 378–455. https://doi.org/10.1210/er.2011-1050

Vargas, A., Moreau, J., Landry, S., LeBellego, F., Toufaily, C., Rassart, É., … Barbeau, B. (2009). Syncytin-2 Plays an Important Role in the Fusion of Human Trophoblast Cells. *Journal of Molecular Biology*, *392*(2), 301–318. https://doi.org/10.1016/j.jmb.2009.07.025

Vernetti, L., Gough, A., Baetz, N., Blutt, S., Broughman, J. R., Brown, J. A., … Taylor, D. L. (2017). Functional Coupling of Human Microphysiology Systems: Intestine, Liver, Kidney Proximal Tubule, Blood-Brain Barrier and Skeletal Muscle. *Scientific Reports*, *7*, 42296. https://doi.org/10.1038/srep42296

Vinatier, D., & Monnier, J. C. (1995). Pre-eclampsia: physiology and immunological aspects. *European Journal of Obstetrics & Gynecology and Reproductive Biology*, *61*, 85–97. Retrieved from https://ac-els-cdn-com.ezproxy.brunel.ac.uk/030121159502106H/1-s2.0-030121159502106H-main.pdf?_tid=cfc20f81-8527-4d15-b3d1-62909e1ae5f9&acdnat=1526478816_f2b4c6c4de797f21403f47acf1514a8f

Vitku, J., Heracek, J., Sosvorova, L., Hampl, R., Chlupacova, T., Hill, M., … Starka, L. (2016). Associations of bisphenol A and polychlorinated biphenyls with spermatogenesis and steroidogenesis in two biological fluids from men attending an infertility clinic. *Environment International*, *89–90*, 166–173. https://doi.org/10.1016/j.envint.2016.01.021

Vlahos, C. J., Matter, W. F., Hui, K. Y., & Brown, R. F. (1994). A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *The Journal of Biological Chemistry*, *269*(7), 5241–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8106507

Vom Saal, F. S., Cooke, P. S., Buchanan, D. L., Palanza, P., Thayer, K. A., Nagel, S. C., … Welshons, W. V. (1998). A Physiologically Based Approach To the Study of Bisphenol a and Other Estrogenic Chemicals On the Size of Reproductive Organs, Daily Sperm Production, and Behavior. *Toxicology and Industrial Health*, *14*(1–2), 239–260. https://doi.org/10.1177/074823379801400115

vom Saal, F. S., Timms, B. G., Montano, M. M., Palanza, P., Thayer, K. A., Nagel, S. C., … Welshons, W. V. (1997). Prostate enlargement in mice due to fetal exposure to low doses of estradiol or diethylstilbestrol and opposite effects at high doses. *Proceedings of the National Acacemy of Science of the United States of America*, *94*(March), 2056–2061. https://doi.org/10.1073/pnas.94.5.2056

Vrtačnik, P., Ostanek, B., Mencej-Bedrač, S., & Marc, J. (2014). The many faces of estrogen signaling. *Biochemia Medica*, *24*(3), 329–42. https://doi.org/10.11613/BM.2014.035

Wakeling, A. E., & Bowler, J. (1992). ICI 182,780, a new antioestrogen with clinical potential. *The Journal of Steroid Biochemistry and Molecular Biology*, *43*(1–3), 173–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1525058

Walsh, C. T., Radeff-Huang, J., Matteo, R., Hsiao, A., Subramaniam, S., Stupack, D., & Brown, J. H. (2008). Thrombin receptor and RhoA mediate cell proliferation through integrins and cysteine-rich protein 61. *The FASEB Journal*, *22*(11), 4011–4021. https://doi.org/10.1096/fj.08-113266

Wang, C., Fu, W., Quan, C., Yan, M., Liu, C., Qi, S., & Yang, K. (2015). The role of Pten/Akt signaling pathway involved in BPA-induced apoptosis of rat Sertoli cells. *Environmental Toxicology*, *30*(7), 793–802. https://doi.org/10.1002/tox.21958

Wang, C., Li, Z., Han, H., Luo, G., Zhou, B., Wang, S., & Wang, J. (2016). Impairment of object recognition memory by maternal bisphenol A exposure is associated with inhibition of Akt and ERK/CREB/BDNF pathway in the male offspring hippocampus. *Toxicology*, *341–343*, 56–64. https://doi.org/10.1016/j.tox.2016.01.010

Wang, H., Pilla, F., Anderson, S., Martinez-Escribano, S., Herrer, I., Moreno-Moya, J. M., … Horcajadas, J. A. (2012). A novel model of human implantation: 3D endometrium-like culture system to study attachment of human trophoblast (Jar) cell spheroids. *Molecular Human Reproduction*, *18*(1), 33–43. https://doi.org/10.1093/molehr/gar064

Wang, Z.-Y., Lu, J., Zhang, Y.-Z., Zhang, M., Liu, T., & Qu, X.-L. (2015). Effect of Bisphenol A on invasion ability of human trophoblastic cell line BeWo. *International Journal of Clinical and Experimental Pathology*, *8*(11), 14355–64. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/26823751

Warner, M., Ye, M., Harley, K., Kogut, K., Bradman, A., & Eskenazi, B. (2017). Prenatal DDT exposure and child adiposity at age 12: The CHAMACOS study. *Environmental Research*, *159*, 606–612. https://doi.org/10.1016/j.envres.2017.08.050

Watkins, D. J., Sánchez, B. N., Téllez-Rojo, M. M., Lee, J. M., Mercado-García, A., Blank-Goldenberg, C., … Watkins, D. J. (2017). Phthalate and bisphenol A exposure during in utero windows of susceptibility in relation to reproductive hormones and pubertal development in girls. *Environmental Research*, *159*, 143–151. https://doi.org/10.1016/j.envres.2017.07.051

Webster, T. F. (2013). Mixtures of endocrine disruptors: How similar must mechanisms be for concentration addition to apply? *Toxicology*, *313*(2–3), 129–133. https://doi.org/10.1016/j.tox.2013.01.009

Welshons, W. V., Nagel, S. C., & Vom Saal, F. S. (2006). Large effects from small exposures. III. Endocrine mechanisms mediating effects of bisphenol A at levels of human exposure. *Endocrinology*, *147*(6), 56–69. https://doi.org/10.1210/en.2005-1159

Wertheimer, E., Gutierrez-Uzquiza, A., Rosemblit, C., Lopez-Haber, C., Sosa, M. S., & Kazanietz, M. G. (2012). Rac signaling in breast cancer: a tale of GEFs and GAPs. *Cellular Signalling*, *24*(2), 353–62. https://doi.org/10.1016/j.cellsig.2011.08.011

Whyatt, R. M., Liu, X., Rauh, V. A., Calafat, A. M., Just, A. C., Hoepner, L., … Factor-Litvak, P. (2011). Maternal Prenatal Urinary Phthalate Metabolite Concentrations

and Child Mental, Psychomotor, and Behavioral Development at 3 Years of Age. *Environmental Health Perspectives*, *120*(2), 290–295. https://doi.org/10.1289/ehp.1103705

Wice, B., Menton, D., Geuze, H., & Schwartz, A. L. (1990). Modulators of cyclic AMP metabolism induce syncytiotrophoblast formation in vitro. *Experimental Cell Research*, *186*(2), 306–316. https://doi.org/10.1016/0014-4827(90)90310-7

Widmer, M., Villar, J., Benigni, A., Conde-Agudelo, A., Karumanchi, S. A., & Lindheimer, M. (2007). Mapping the Theories of Preeclampsia and the Role of Angiogenic Factors. *Obstetrics & Gynecology*, *109*(1), 168–180. https://doi.org/10.1097/01.AOG.0000249609.04831.7c

Winn, V. D., Haimov-Kochman, R., Paquet, A. C., Yang, Y. J., Madhusudhan, M. S., Gormley, M., … Fisher, S. J. (2007). Gene expression profiling of the human maternal-fetal interface reveals dramatic changes between midgestation and term. *Endocrinology*, *148*(3), 1059–1079. https://doi.org/10.1210/en.2006-0683

Witorsch, R. J. (2002). Endocrine disruptors: Can biological effects and environmental risks be predicted? *Regulatory Toxicology and Pharmacology*, *36*(1), 118–130. https://doi.org/10.1006/rtph.2002.1564

Wolfe, M. W. (2006). Culture and transfection of human choriocarcinoma cells. *Methods in Molecular Medicine*, *121*, 229–239.

Xiao, S., Diao, H., Smith, M. A., Song, X., & Ye, X. (2011). Preimplantation exposure to bisphenol A (BPA) affects embryo transport, preimplantation embryo development, and uterine receptivity in mice. *Reproductive Toxicology*, *32*(4), 434–441. https://doi.org/10.1016/j.reprotox.2011.08.010

Xu, X., Lu, Y., Zhang, G., Chen, L., Tian, D., Shen, X., … Dong, F. (2014). Bisphenol A promotes dendritic morphogenesis of hippocampal neurons through estrogen receptor-mediated ERK1/2 signal pathway. *Chemosphere*, *96*, 129–37. https://doi.org/10.1016/j.chemosphere.2013.09.063

Yang, Q., Zhao, Y., Qiu, X., Zhang, C., Li, R., & Qiao, J. (2015). Association of serum levels of typical organic pollutants with polycystic ovary syndrome (PCOS): a case–control study. *Human Reproduction*, *30*(8), 1964–1973. https://doi.org/10.1093/humrep/dev123

Yang, R., Tao, Z.-Z., Huang, M.-L., Zheng, Y.-F., Dai, M.-Y., Zou, Y., & Chen, S.-M. (2018). Knockout of the placenta specific 8 gene radiosensitizes nasopharyngeal carcinoma cells by activating the PI3K/AKT/GSK3β pathway. *American Journal of Translational Research*, *10*(2), 455–464. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/29511439

Yap, A. S., & Kovacs, E. M. (2003). Direct cadherin-activated cell signaling: a view from the plasma membrane. *The Journal of Cell Biology*, *160*(1), 11–6. https://doi.org/10.1083/jcb.200208156

Yashwanth, R., Rama, S., Anbalagan, M., & Rao, A. J. (2006). Role of estrogen in regulation of cellular differentiation: A study using human placental and rat Leydig cells. *Molecular and Cellular Endocrinology*, *246*(1–2), 114–120.

https://doi.org/10.1016/J.MCE.2005.11.007

Ye, M., Beach, J., Martin, J. W., & Senthilselvan, A. (2016). Pesticide exposures and respiratory health in general populations. *Journal of Environmental Sciences*, *51*, 361–370. https://doi.org/10.1016/j.jes.2016.11.012

Yin, G., Zhu, X., Guo, C., Yang, Y., Han, T., Chen, L., … Liang, L. (2013). Differential expression of estradiol and estrogen receptor α in severe preeclamptic pregnancies compared with normal pregnancies. *Molecular Medicine Reports*, *7*(3), 981–985. https://doi.org/10.3892/mmr.2013.1262

Yolton, K., Xu, Y., Strauss, D., Altaye, M., Calafat, A. M., & Khoury, J. (2011). Prenatal exposure to bisphenol A and phthalates and infant neurobehavior. https://doi.org/10.1016/j.ntt.2011.08.003

Yorita Christensen, K. L., & White, P. (2011). A methodological approach to assessing the health impact of environmental chemical mixtures: PCBs and hypertension in the National Health and Nutrition Examination Survey. *International Journal of Environmental Research and Public Health*, *8*(11), 4220–37. https://doi.org/10.3390/ijerph8114220

Yu, J. S. L., & Cui, W. (2016). Proliferation, survival and metabolism: the role of PI3K/AKT/mTOR signalling in pluripotency and cell fate determination. *Development (Cambridge, England)*, *143*(17), 3050–60. https://doi.org/10.1242/dev.137075

Yu, O. M., & Brown, J. H. (2015). G Protein-Coupled Receptor and RhoA-Stimulated Transcriptional Responses: Links to Inflammation, Differentiation, and Cell Proliferation. *Molecular Pharmacology*, *88*(1), 171–80. https://doi.org/10.1124/mol.115.097857

Yudilevich, D. L., & Barros, L. F. (1990). Transport of amino acids and nucleosides in the placenta. *Biochemical Society Transactions*, *18*(6), 1136–40. https://doi.org/10.1042/BST0181136

Yura, S., Sagawa, N., Itoh, H., Kakui, K., Nuamah, M. A., Korita, D., … Fujii, S. (2003). Resistin Is Expressed in the Human Placenta. *The Journal of Clinical Endocrinology & Metabolism*, *88*(3), 1394–1397. https://doi.org/10.1210/jc.2002-011926

Zachariades, E., Mparmpakas, D., Pang, Y., Rand-Weaver, M., Thomas, P., & Karteris, E. (2012). Changes in placental progesterone receptors in term and preterm labour. *Placenta*, *33*(5), 367–372. https://doi.org/10.1016/j.placenta.2012.01.002

Zandvakili, I., Lin, Y., Morris, J. C., & Zheng, Y. (2017). Rho GTPases: Anti- or pro-neoplastic targets? *Oncogene*, *36*(23), 3213–3222. https://doi.org/10.1038/onc.2016.473

Zhang, H.-Q., Zhang, X.-F., Zhang, L.-J., Chao, H.-H., Pan, B., Feng, Y.-M., … Shen, W. (2012). Fetal exposure to bisphenol A affects the primordial follicle formation by inhibiting the meiotic progression of oocytes. *Molecular Biology Reports*, *39*(5), 5651–5657. https://doi.org/10.1007/s11033-011-1372-3

Zhang, Y., Wei, F., Zhang, J., Hao, L., Jiang, J., Dang, L., … Jiang, L. (2017). Bisphenol A and estrogen induce proliferation of human thyroid tumor cells via an estrogen-receptor-dependent pathway. *Archives of Biochemistry and Biophysics*, *633*, 29–39. https://doi.org/10.1016/j.abb.2017.09.002

Zhang, Z., Zhang, L., Jia, L., Cui, S., Shi, Y., Chang, A., … Wang, P. (2013). AP-2α suppresses invasion in BeWo cells by repression of matrix metalloproteinase-2 and -9 and up-regulation of E-cadherin. *Molecular and Cellular Biochemistry*, *381*(1–2), 31–39. https://doi.org/10.1007/s11010-013-1685-8

Zhao, L.-C., Shen, B.-Y., Deng, X.-X., Chen, H., Zhu, Z.-G., & Peng, C.-H. (2016). TMEM45B promotes proliferation, invasion and migration and inhibits apoptosis in pancreatic cancer cells. *Molecular BioSystems*, *12*(6), 1860–70. https://doi.org/10.1039/c6mb00203j

Zhou, L., Chen, H., Mao, X., Qi, H., Baker, P. N., & Zhang, H. (2017). G-protein-coupled receptor 30 mediates the effects of estrogen on endothelial cell tube formation in vitro. *International Journal of Molecular Medicine*, *39*(6), 1461–1467. https://doi.org/10.3892/ijmm.2017.2957

Zhou, Q., Hao, L., Huang, W., & Cai, Z. (2016). The Golgi-Associated Plant Pathogenesis-Related Protein GAPR-1 Enhances Type I Interferon Signaling Pathway in Response to Toll-Like Receptor 4. *Inflammation*, *39*(2), 706–717. https://doi.org/10.1007/s10753-015-0297-8

Zhou, S., Yu, D., Ning, S., Zhang, H., Jiang, L., He, L., … Sun, M. (2015). Augmented Rac1 Expression and Activity are Associated with Oxidative Stress and Decline of β Cell Function in Obesity. *Cellular Physiology and Biochemistry : International Journal of Experimental Cellular Physiology, Biochemistry, and Pharmacology*, *35*(6), 2135–48. https://doi.org/10.1159/000374019

Zhou, Y., Zhang, M., Guo, W., Yu, M., Xue, K., Huang, S., … Guo, T. (2006). Expression of resistin protein in normal human subcutaneous adipose tissue and pregnant women subcutaneous adipose tissue and placenta. *Journal of Huazhong University of Science and Technology. Medical Sciences = Hua Zhong Ke Ji Da Xue Xue Bao. Yi Xue Ying De Wen Ban = Huazhong Keji Daxue Xuebao. Yixue Yingdewen Ban*, *26*(3), 288–91. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16961271

Zhu, G., Huang, Y., Wu, C., Wei, D., & Shi, Y. (2016). Activation of G-Protein-Coupled Estrogen Receptor Inhibits the Migration of Human Nonsmall Cell Lung Cancer Cells via IKK-β/NF-κB Signals. *DNA and Cell Biology*, *35*(8), 434–442. https://doi.org/10.1089/dna.2016.3235

Zou, L., Chai, J., Gao, Y., Guan, J., Liu, Q., & Du, J.-J. (2016). Down-regulated PLAC8 promotes hepatocellular carcinoma cell proliferation by enhancing PI3K/Akt/GSK3β/Wnt/β-catenin signaling. *Biomedicine & Pharmacotherapy*, *84*, 139–146. https://doi.org/10.1016/j.biopha.2016.09.015

Zung, A., Glaser, T., Kerem, Z., & Zadik, Z. (2008). Breast development in the first 2 years of life: An association with soy-based infant formulas. *Journal of Pediatric Gastroenterology and Nutrition*, *46*(2), 191–195. https://doi.org/10.1097/MPG.0b013e318159e6ae

Zwezdaryk, K. J., Warner, J. A., Machado, H. L., Morris, C. A., & Höner zu Bentrup, K. (2012). Rotating Cell Culture Systems for Human Cell Culture: Human Trophoblast Cells as a Model. *Journal of Visualized Experiments*, (59). https://doi.org/10.3791/3367

Appendix

HES7 GATAD2A EML1 MYL3 GAGE7 ATF3 DPYSL2 PPARGC1B VPS37D FAT3 CDCA4 LYPD4 LINC00963 HRK SLC7A4 EXTL2 MRPL52 FRYL THAP9-AS1 RASL10A KANK1 C1ORF226 MDM4 FBXO27 KRCC1 CCDC144A FNTB LOC101927070 MBP BMF ATP6V0E2-AS1 ZNF641 ITGA1 GFPT1 MSL3 CDR2L SAR1A KIAA1522 FAM209A TBX3 TRIM36 RFPL4AL1 AGXT C1S EFR3B UPP1XLOC_L2_010056 UNKL PCDH1 RTCA STX16 RAB11FIP1 EAF2 ANK3 NKAIN4 NUDCD3 C7ORF55 MST1 PER2 NLRP12 TCEA1 TNFRSF13C ICA1 OPHN1 ANKRD13A DCLRE1C NEXN SLC1A3 UBE3B CLGN SGK494 SLC30A2 ACTG2 HCAR3 TGM1 BCKDHB CAV1 TMEM45AT MEM45B DDX46 WNT6 TAC4 LEPREL2 ALS2 ACIN1DYNLT1 TRIB3SIGIRR FAM178A ZC2HC1A PCDHB2 TNFSF4 GABPB1 BACH2 APOL6PRTG CREB3L3 SNAI3 NGLY1 PCOLCE-AS1PLAC8 MIOX WDR33 TMSB15A GPR124 CDKN2C SERPINB9 RNF125 LINC00869 PLIN5 LINC00867 RNF121 HBEGF SPEF1 LPCAT2 ARFRP1 ENSA ANKRD36B DGKD LAMP3 SLC39A7 OSBPL7 FANCC KIFC1 PITPNM3 ZNF778 TCAM1P MAP3K13 ANKRD33B CLSTN3 INSIG2 AK3 C1ORF54 CBLN1 DHCR24 PTPN14 KBTBD11 DNAJC1 SOS2 EIF4E2 CATSPER2 NFAT5 C3ORF52 PIP5KL1 LNC-RALGAPA1-3 DRD5 LINC01301 MLXIP TNRC6C-AS1 LINC01061 ASS1 LEP UHRF1BP1 PFN2 C14ORF79 TBL1X TBL1Y

Table 1. Shared differentially regulated genes in 3 nM and 10n M BPA treated non-syncytialised BeWo cells