Essential Signaling Cascades as Predictive Endpoints for Teratogenicity *in vitro*:

A Proof of Principle Study

Inaugural-Dissertation

To obtain the academic degree

Doctor rerum naturalium (Dr. rer. nat.)

Submitted to the Department of Biology, Chemistry, and Pharmacy

of Freie Universität Berlin

by

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From Plauen, Germany

2016

Die vorliegende Arbeit wurde auf Anregung und unter Anleitung von Prof. Dr. Dr. Andreas Luch in der Zeit von August 2011 bis Juli 2016 am Bundesinstitut für Risikobewertung, Berlin, angefertigt.

1. Gutachter: Prof. Dr. Dr. Andreas Luch (Freie Universität Berlin)
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Disputation am: 21.11.2016

Erklärung

Hiermit versichere ich, die vorliegende Arbeit mit dem Titel „Essential Signaling Cascades as Predictive Endpoints for Teratogenicity *in vitro*: A Proof of Principle Study“ ohne Benutzung anderer als der zugelassenen Hilfsmittel selbstständig angefertigt zu haben. Alle angeführten Zitate sind als solche kenntlich gemacht. Die vorliegende Arbeit wurde in keinem früheren Promotionsverfahren angenommen oder als ungenügend beurteilt.

Berlin, Juli 2016

Die Dissertation wurde in englischer Sprache verfasst.

Content

1. Introduction

Mammalian embryonic development is a very complex and fascinating process, in which all cell types, organs and complex structures of the embryo and the later adult are generated out of one fertilized cell. This process is considered particularly susceptible to external interferences that can induce a wide variety of severe, teratogenic effects that eventually lead to embryonic lethality. The present work focused on the development of *in vitro* test systems to predict teratogenicity based on the qualitative and quantitative analysis of conserved signaling cascades that are essential components of the regulatory network controlling embryonic development.

First the term teratogenicity and testing strategies for teratogens shall be elucidated, thereby contrasting established and accepted *in vivo* tests and *in vitro* alternatives. Since most alternatives rely on embryonic stem cells, their function and utilization will be discussed focusing on the differentiation of stem cells into cardiomyocytes that lays the basis for the well established embryonic stem cell test, which served as a reference test for this work. The second aspect, essential signaling cascades involved in embryonic development will be depicted in the following section focusing on the role of Bmp and Wnt signaling in embryonic development and cellular differentiation. Within this section, I will also describe the two different transgenic reporter gene mouse models, from which the embryonic stem cell lines that I used in the present study originated. As a third important aspect, the substances used to characterize the test systems and questions raised will be examined.

1.1 Teratology

1.1.1 General Aspects and present testing strategies

Teratology is defined as the science that studies the causes, mechanisms, and patterns of abnormal development. This includes congenital malformations, growth retardation, fetal death, and functional defects that occur with a prevalence among all live births and stillbirths of about 3 to 4 % (Peters et al. 2015). Several factors can cause birth defects, some of which are well understood, in particular monogenetic conditions and chromosomal disorders, with an incidence of 20 and 5 % of all congenital disorders, respectively (Schaefer 2005). However, in 65 % of all birth defects the cause is unknown or considered to be caused by a combination of multiple factors

Developmental toxicity analyzes all functional and structural alterations occurring during the development of an organism from fertilization until puberty that are caused through environmental factors (Rogers and Kavlock 1998). In general, the incidence of teratogen caused perturbations lies at 2 to 8.5 % of all

congenital disorders and comprises chemicals, drugs, life-style such as alcohol, environmental toxic substances, but also physical factors (Peters et al. 2015).

Already in 1977, Wilson described principles for drug-induced reproductive and developmental toxicology (Wilson 1977). First there are species differences in the susceptibility to a given teratogen as exemplified by thalidomide that causes malformations in primates, rabbits, frogs, hamster, chicken and even zebrafish, but not in rat or mice (Vargesson 2015). Thus, two different animal species have to be tested for adverse effects in most cases. Another important aspect is the time point of exposure during development. The process can be divided into three phases: The embryonic, fetal and postnatal periods (Figure 1). During the embryonic period, the fertilized egg undergoes several cell divisions until implantation and first specialization separates embryonic from extraembryonic tissues that later form parts of the placenta and the umbilical cord. After implantation, the three germ layers, ectoderm, mesoderm and endoderm are formed that give rise to various tissues and organs. In general this period is the most susceptible time in which exogenic factors have the highest impact on the developing organism. Until implantation the cells are pluripotent and can differentiate into all cells of the developing organisms. Therefore toxic effects result either in embryonic lethality and abortion or resorption, or if these effects can be compensated have no effect. After implantation, the cells differentiate into mesoderm, endoderm, and ectoderm, allowing cell type specific effects of toxic substances. Similarly, every organ has its own susceptible period specified through the onset of differentiation and various phases of organogenesis. With ongoing development the severity of effects caused by teratogens decreases, for example during the fetal period which is defined by further tissue growth and functional maturation. Since functional maturation is not finished at birth, but takes also place during the postnatal phase until puberty, this period is also included in the analysis of developmental toxicology. Finally dosage is also an important aspect that needs to be considered. The severity of effects will increase with dose and, thus, it is often also possible to determine concentrations at which the agent has no effect – the No Observed Adverse Effect Level (NOAEL). However, effects on the embryo should be detectable without any signs of toxicity in the mother, since, otherwise, potentially specific, embryotoxic effects can not be separated from indirect effects mediated by the loss of function of maternal tissues. Especially for teratogens the dose-response curves are very steep and often overall (maternal) toxic concentrations are close to teratogenic concentrations. It can be expected that the adverse effect itself, but also the critical time period and potential species differences are dependent on the mode of action (MoA) of the teratogen, and, thus, a better understanding of the molecular mechanism mediating embryotoxic activities are an important aspect of developmental toxicology.

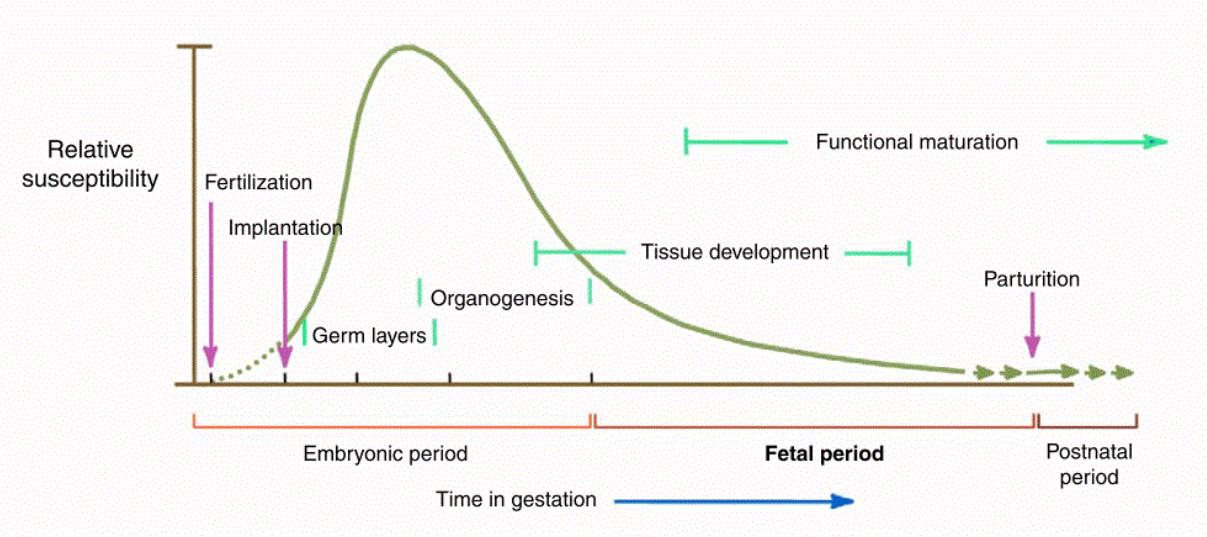

Figure 1: Change of susceptibility during development: From fertilization until implantation, the cells are pluripotent and toxic agents have either no effect or lead to embryonic lethality. After implantation the three germ layer form and organogenesis starts. During this period the developing embryo is the most susceptible and exposure to teratogens results in major birth defects. The fetal period is characterized through tissue development and functional maturation, such that the exposure to teratogens only results in minor defects. Effects of teratogens during postnatal period have to be considered as well (modified from DeSesso 2010).

Presently the testing on developmental toxicology relies on experimental animals. Since 1981, the Organisation for Economic Co-operation and Development (OECD) and partner countries developed standardized test guidelines for the testing of chemicals. The testing for teratogenic activities according to OECD Guidelines relies on repeated treatment of experimental animals prior to and during pregnancy and analyzes adverse effects in the pups, as described in the prenatal development toxicity study (OECD TG 414, OECD 2001), the reproduction/ developmental toxicity screening test (OECD TG 421, OECD 2015), and the combined repeated dose toxicity study with the reproduction/ developmental toxicity screening test (OECD TG 422, OECD 2015b). Because of the anticipated species differences in susceptibility, the testing has to be carried out in a rodent and a non-rodent model with at least three different dosages that allow the determination of a NOAEL. All of these methods require a high number of experimental animals, are very time-consuming and expensive (Fleischer 2007).

1.1.2 In vitro alternatives and recent developments

Two major changes in legislation increased the need for *in vitro* alternatives: (1) for cosmetic products the EU banned the marketing of ingredients tested in animals in 2013 (European Commission 2009). (2) Under the new chemical legislation REACH (Registration, Evaluation, Authorisation, and Restriction of Chemicals) all chemical substances with a production volume from one ton per year (t/a) have to be registered, tested,

and evaluated until 2018 (Parliament and the Council of the European Union 2006). Although one scope of REACH is to promote alternative methods for risk assessment, Van der Jagt et al. (2004) predicted the need of 240 000 additional animals per year for the prediction of teratogenic activities alone. In the last years different approaches for *in vitro* alternatives for developmental toxicity became apparent: (1) the usage of whole-embryos or cells thereof in *ex vivo* preparations, (2) the differentiation of embryonic stem cells into different cell types, and (3) the combination of high through-put assays for the identification of toxicity pathways.

Already in 2004 the European Centre for the Validation of Alternative Methods (ECVAM) validated three different *ex vivo* and *in vitro* test systems, the Micromass Test (MM, Spielmann et al. 2004), the Whole-Embryo Culture Test (WEC, Piersma et al. 2004), and the Embryonic Stem Cell Test (EST, Genschow et al. 2004). In the WEC, post-implantation rat embryos are cultured for 48 h in the presence of test substances. During this period, a beating heart and circulation, as well as different organ anlagen will develop and the neural tube will close. The morphology of the embryo is scored and used to predict teratogenicity of the substances applied (Brown and Fabro 1981). For the MM test, cells of the developing limb anlagen of day 14 rat embryos are prepared, cultured for five days, and differentiated into chondrocytes. The teratogenic potential of the test substance is evaluated by the comparison of the effect on cell viability (IC_{50}) and on the differentiation (ID_{50}, Flint et al. 1984). During the validation, the described tests reached an accuracy of 80 % for the WEC and 70 % for the MM test when compared with *in vivo* data. However, for both tests pregnant animals have to be sacrificed. In contrast, the EST utilizes embryonic stem cells that can be propagated indefinitely after isolation from early blastocyst stage embryos. The EST benefits from the pluripotent nature of the embryonic stem cells, which are differentiated into cardiomyocytes within ten days. The success of the differentiation can be monitored by light microscopy in which spontaneously beating cardiomyocytes are observed. The impact of teratogens on the differentiation (ID_{50}) is compared to effects on the cell viability of the embryonic stem cells (IC_{50} D3) and somatic NIH3T3 cells (IC_{50} 3T3). Although the accuracy of the EST was evaluated with 78 % (Genschow et al. 2004), its acceptance as alternative for the prediction of teratogenicity requires further improvements. The main critique alludes to the fact that only one route of differentiation is analyzed, such that additional endpoints covering other routes of differentiation and cell types should be included for regulatory acceptance (Marx-Stoelting et al. 2009).

Over the last years, several protocols for differentiation of embryonic stem cells into distinct lineages establishing new endpoints, were deployed as *in vitro* test systems, including differentiation into neurons (Hayess et al. 2013), bone (Kuske et al. 2012; Sittner et al. 2016), and endothelial cells (Festag et al. 2007a).

To analyze the differentiation process these protocols rely on the quantification of specific marker gene expression, either on the transcriptional or the translational level, making them more laborious and prone to errors. Nevertheless, comparison of general cytotoxicity with the inhibition of differentiation is a recurring method, as teratogens tend to influence specifically the differentiation in concentrations not yet being cytotoxic. Easier detection of biological processes is possible with transgenic modified cells. Uibel et al. (2010) modified mouse embryonic stem cells to express a reporter gene under the influence of active Wnt/β-Catenin signaling, an essential signaling cascade for the development and differentiation (compare section 1.3.3), and demonstrated that teratogens can alter the transcription of the reporter gene in undifferentiated stem cells without exhibiting cytotoxic effects.

Another approach combines several high-throughput assays with computational analysis. Two independent research programs use the combined data of diverse *in vitro* assays with several endpoints to predict toxic effects of tested compounds, the ToxCast Research program of the US Environmental Protection Agency (EPA) and the Tox21 consortium (Collins et al. 2008; Dix et al. 2007). Alone in the ToxCast program phase I, over 300 chemicals were tested in over 600 assays (Kavlock et al. 2012). Using this approach, Kleinstreuer et al. (2011) could establish an *in silico* model to predict developmental vascular toxicity, proofing the potential of the collected data and underlining that such programs can help to understand the mode of action of possible teratogens.

In general all described *in vitro* alternatives are not regulatory accepted and, because of the complexity of development, not one single test will suffice to predict teratogenicity (Adler et al. 2011). But it can also be concluded that embryonic stem cells are a promising tool, as they model embryonic development faithfully *in vitro*. The biological characteristics of embryonic stem cells shall be further explained in the following section.

1.2 Embryonic stem cells

1.2.1 Characteristics and usage

The first description of the successful cultivation of mouse embryonic stem cells (ESCs) was in 1981 by Evans and Kaufman (Evans and Kaufman 1981) who laid the basics for the establishment of stem cell research that provided an invaluable tool for the analysis of basic developmental processes, as well as the generation of a large and still increasing number of genetically modified mouse lines allowing the molecular analysis of gene function and human disease *in vivo* and *in vitro*.

The concept of pluripotent stem cells was established over decades (Solter 2006) and is based on the analysis of teratocarcinomas that can be found with a rather high incidence in some mouse strains, e.g. the 129 mouse line and derivatives thereof (Stevens 1973). These tumors are characterized by the formation of all kinds of tissues, including bone, hair, and teeth. Cell lines isolated from these tumors can be grown in culture indefinitely and single teratocarcinoma cells will induce new tumors composed again of all kind of tissues after transplantation into recipient mice demonstrating a pluripotent character (Kleinsmith and Pierce 1964). Similarly, transplantation of early embryos can also induce teratocarcinomas from which again teratocarcinoma cell lines can be established (Solter et al., 1970). These experiments implicated that pluripotent cells in teratocarcinomas and embryos are highly similar and let to various attempts to isolate pluripotent cells directly from mouse embryos that were finally successful in the early 1980s.

For the preparation of ESCs the time-point is crucial. After fertilization the cells undergo several cell divisions to form the morula stage and subsequently, still before implantation, the embryo develops into the blastocyst with a inner cell mass (ICM), later giving rise to the embryo proper, and an outer cell layer, the trophoblast, further developing into extra-embryonic tissues. Although, the first ESCs were isolated from the epiblast of delayed-implantation blastocysts, cells are now derived from the inner cell mass cells of blastocysts from embryonic day 4 (E4.0), as described by Doetschman et al. (1985) and subsequently cultivated on so-called feeder cells, mitotically inactivated (mitomycin C-treated) embryonic fibroblasts. This protocol is still commonly used, because it ensures that the cells retain the capacity to differentiate into all three germ layers as well as germ cells (see figure 2). The three germ layers, endoderm, mesoderm, and ectoderm, can form all functional cell types of the adult organism. In contrast to other primary cell cultures, ESCs can be propagated for indefinite time period in which they undergo symmetrical cell division without showing evidence of senescence or crisis and retain the pluripotent capability to differentiate in all three germ layer *in vitro* as well as *in vivo* (Suda et al. 1987).

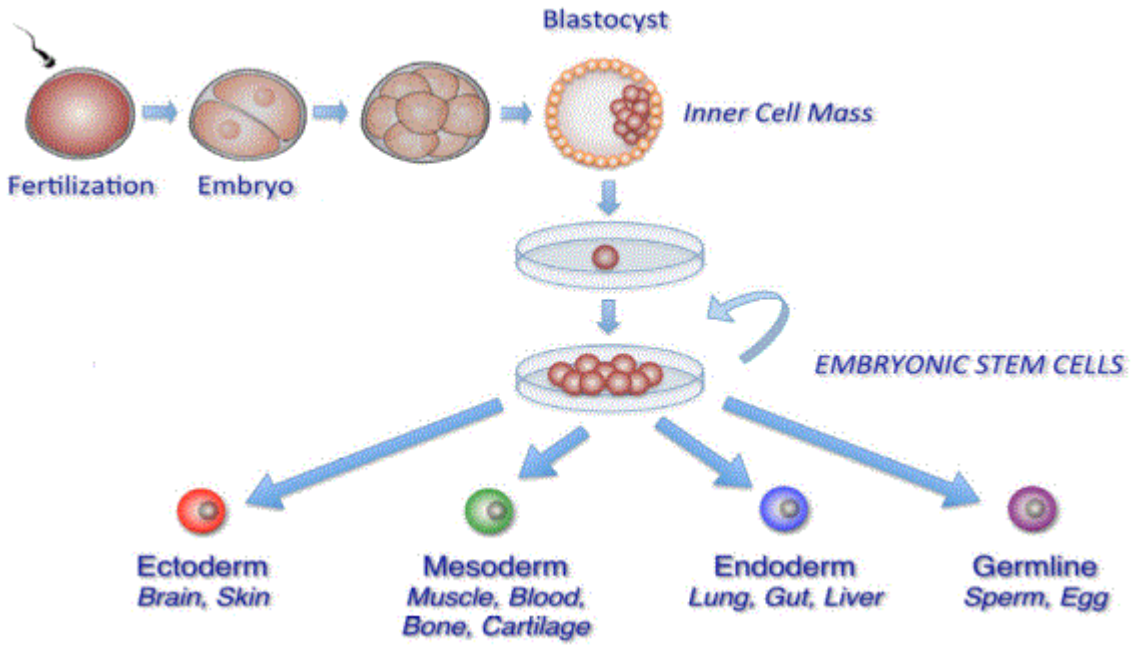

Figure 2: Generation and pluripotent character of embryonic stem cells (ESCs). After several cell divisions the blastocyst forms, with the inner cell mass (ICM), giving raise to the three germ layers, and the surrounding trophoblast, forming the extraembryonic tissues. Around embryonic day 4.0 (E4.0) the blastocysts are gathered and the cells cultured on feeder cells keeping the ESCs pluripotent, such that they can form the three germ layer – ectoderm, mesoderm, and endoderm – as well as germ cells (modified from Yabut and Bernstein 2011).

During the cultivation, ESCs can either undergo symmetrical division and grow, staying pluripotent, or commit to a cellular lineage and differentiate. For example, removal of the feeder cell layer promotes the loss of pluripotency and the induction of differentiation. In 1988 the identification of the cytokine Leukemia Inhibitory Factor (LIF) enabled the cultivation of ESCs without feeder cells (Smith et al. 1988; Williams et al. 1988) and laid the basis for the discovery of the complex network regulating pluripotency (Martello and Smith 2014). Along with LIF, signaling proteins of the bone morphogenetic protein family (Bmp, see also section 1.3.2) originating from the fetal calf serum (FCS) play an essential role in the regulation of pluripotency (see figure 3A). A combination of Bmp and LIF signaling suffices to inhibit differentiation of the ESCs such that they stay pluripotent. The activity of Bmp is at least partially mediated by the stimulation of expression of Id (Inhibitor of Differentiation or Inhibitor of DNA binding) transcription factors. LIF signals through the LIF receptor (LIFRβ) and gp130 inducing growth and cell survival and inhibiting differentiation (see figure 3A). In 2008, Ying *et al.* described the use of chemical inhibitors to stabilize what they called the "the ground state of pluripotency". Addition of only two small chemical inhibitors, CHIR99021 und PD184352 , inhibits cellular commitment efficiently and stabilized growth and viability of pluripotent cells (see Figure 3B, Ying et al. 2008). CHIR99021 simulates active Wnt signaling

through inhibition of glycogen synthase kinase 3β (Gsk3β, see also section 1.3.3), thereby positively influencing growth and viability whereas PD0325901 inhibits the activity of MAPK kinases (MEK) that mediate signal transduction through receptor tyrosine kinases, like FGF receptors and prevents cellular differentiation.

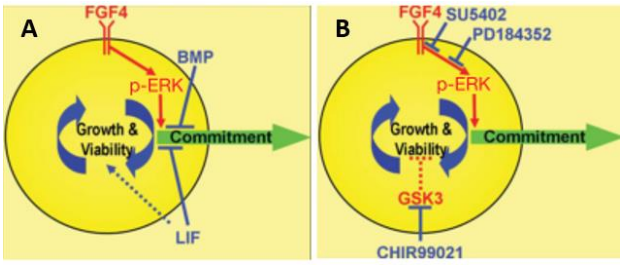

Figure 3: Signaling pathways involved in the maintenance of pluripotency. Schematic representation of ESCs with the possibility to maintain pluripotency or to commit to cell differentiation through different signaling pathways. (A) Through signals from LIF and Bmp cells are hindered to commit to cell differentiation and are stimulated to growth. (B) Identical output of maintaining pluripotency can be reached via stimulation of Wnt signaling due to inhibition of Gsk3β and Fgf4 signaling, induced with two inhibitors (2i, modified from Ying et al. 2008).

The precise mechanism by which pluripotency is maintained or differentiation is induced are still under debate, but it is agreed that the main transcription factors regulating pluripotency are Oct4 encoded by the gene Pou5f1 (the POU domain, class 5, transcription factor 1), Nanog, and Sox2 (Niwa 2007), in which Oct4 is discussed as the central factor in a complex regulatory network (van den Berg et al. 2010). Transcription factors bind regulatory elements in the DNA within the cell nucleus and regulate the expression of target genes, thereby often acting as down stream mediators of extracellular signaling cascades. Oct4 is essential *in vivo* for the formation of the ICM and is down regulated upon further differentiation (Nichols et al. 1998). *In vitro* an intermediate expression level of Oct4 maintains pluripotency, increase as well as decrease in expression level induce differentiation (Niwa et al. 2000; Shimozaki et al. 2003). Oct4 can activate as well as deactivate gene transcription depending on the presence of specific cofactors (Niwa 2001). Together with other transcription factors Oct4 forms the core of the pluripotency circuit, which is regulated by driver signaling pathways (Pardo et al. 2010). The discovery of the essential components of this regulatory network also allowed the derivation and characterization of induced pluripotent stem cells (iPSCs; Takahashi and Yamanaka 2006; Wernig et al. 2007). Nevertheless, the mechanisms regulating pluripotency and differentiation are still under investigation.

ESCs possess some features making them a prominent tool in basic but also translational research. To better elucidate the first steps of development two different approaches can be seen. First the

investigation of cellular differentiation of ESCs into various tissues identifying and characterizing important signaling cascades but also transcription factors, as well as exogenous factors. Second the genetic manipulation in gain- or loss-of-function variants of certain proteins or the introduction of reporter genes, making it possible to unravel the precise expression, function, and eventually even mechanisms of action of the proteins under investigation (Martello and Smith 2014). Importantly, ESCs have been used already for decades to generate genetically modified animals for *in vivo* studies to unravel molecular mechanisms or to generate models of human diseases that can also play an important role in toxicological studies (Boverhof et al. 2011; Capecchi 1989; Maggi and Ciana 2005). Another aspect is the translation of gathered information from mouse ESCs on human ESCs, tissue specific stem cells, and iPSCs as tool for regenerative medicine and the elucidation of specific molecular mechanisms of genetic disorders (Brennand et al. 2014; Butzke et al. 2013; Smith 2001).

In the following section the differentiation of ESCs into cardiomyocytes will be discussed in comparison to heart development *in vivo*.

1.2.2 Differentiation of ESCs into cardiomyocytes

The differentiation from ESCs into cardiomyocytes as well as heart development is a multistep process. Differentiation and development are characterized by changes in marker genes induced by highly conserved essential signaling cascades, in particular members of the Tgfβ (transforming growth factor-β superfamily (including Bmp and Nodal), Wnt, Fgf (fibroblast growth factor), and Shh (sonic hedgehog) (Loebel et al. 2003). Two pathways of central importance, the Wnt and the Bmp pathways will be discussed in detail in the following section. For now, the main focus will lay on marker genes and transcription factors that define a certain cell type during the differentiation with the crucial signals and the sequence in which these occur.

The heart is the first functional organ formed in the developing embryo and the differentiation into cardiomyocytes *in vitro* can also occur spontaneously. Nevertheless, the process is very complex and sensitive to genetic and environmental disturbances, resulting in about 1 % of all live births with cardiac anomalies in humans (Bruneau 2008). The cardiac differentiation can roughly be separated into three steps: (1) The induction of the mesodermal cell fate, (2) followed by the differentiation into cardiac mesoderm, and (3) the final functional specialization into cardiomyocytes.

In the *in vivo* situation the spatiotemporal organization within the embryo defines the signals acting on the cells. At the blastula stage the embryo has already differentiated into extraembryonic trophectoderm and the inner cell mass that separates into ICM and the overlaying primitive endoderm. The first sign of

asymmetry can be observed after implantation when the process called gastrulation is initiated, the morphogenetic process in which the three germ layers are formed. Here a thickening of the primitive ectoderm (the former ICM) at the future posterior end of the embryo becomes apparent which was first described by Hensen in 1876 in the rabbit embryo and is now referred to as the “node” or “Hensen’s node” (Hensen 1876). In the node, the cells undergo Wnt and Bmp dependent epithelial to mesenchymal transition (EMT) and migrate between the ectoderm and endoderm to form the mesoderm. The mesodermal cells migrate in anterior and medial direction to the sites of further organogenesis (Gadue et al. 2005). Importantly, at the same time, inhibitors of the Nodal/Activin, Wnt, and Bmp pathway, called Lefty, Dickkopf, and Chordin, respectively, are secreted locally in the anterior endoderm or the node itself to generate signaling activity gradients throughout the embryo that provide the embryonic cells with positional information (Niehrs 2004).During gastrulation, the most prominent marker gene for mesoderm is a highly conserved gene called Brachyury, a T-box transcription factor which is expressed in all early mesodermal cells and widely used as a marker for mesoderm formation in vertebrates (Kispert and Herrmann 1994; Technau 2001).

One recurring process in development is the epithelial-to-mesenchymal transition (EMT) as well as the reverse process mesenchymal-to-epithelial transition (MET) which are conserved and essential for organogenesis and embryogenesis (Chen et al. 2012). During EMT, the cells change their morphology and behavior. In an epithelial phenotype the cells are organized in sheets with tide intercellular junctions and form a basal layer also defining an apicobasal cell polarity. Upon EMT the cells change their expression profile in particular in respect to cell adhesion molecules of the Cadherin family, loose the tide cell-cell junctions, gain a front-rear polarity, and become invasive. With that the cells prefer cell-ECM (extracellular matrix) contact instead of cell-cell contact (reviewed in Lim and Thiery 2012). The first EMT in development is gastrulation and can be monitored by the expression of Snail which can also be detected during *in vitro* ESC differentiation.

In vitro the same signaling cascades, Bmp and Wnt, initiate mesoderm induction. The differentiation protocol for the cardiomyocyte differentiation utilizes the hanging drop method in which ESCs can form aggregates, so called embroid bodies (EBs), which resemble the early embryo (Wobus et al. 1991). In the EBs the three germ layers are formed as well, although not as precisely organized as in the embryo.

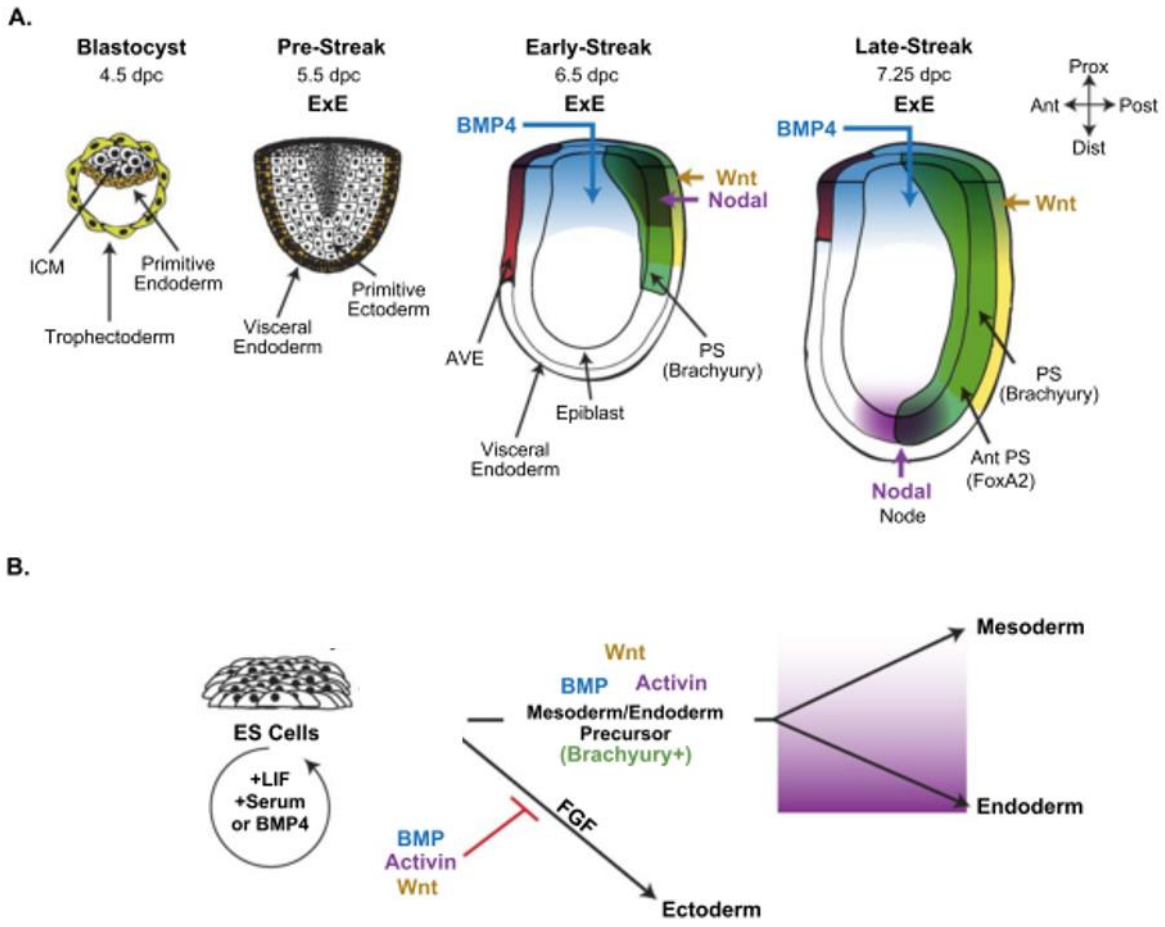

Figure 4: Induction of the three germ layer during gastrulation *in vivo* and *in vitro*. (A) Embryo and surrounding tissues establish gradients of Tgfβ (Nodal), Wnt, and Bmp signaling by which the posterior (post) and anterior (ant) part of the embryo are defined. At the posterior part of the embryo the primitve streak (PS) is established, marked by the expression of Brachyury. The cells of the PS undergo EMT and start to move through the embryo to the sites of later organogenesis. (B) The same signaling cascades induce the differentiation of ESCs into mesoderm and endoderm, in which a gradient of Tgfβ signaling (Nodal or Activin) can determinates the cell fate between mesoderm and endoderm. (modified from Gadue et al. 2005). Prox – proximal, Dist – distal, dpc – days post coitum, ExE – Extraembryonic ectoderm, AVE – anterior viscerial endoderm, ICM – inner cell mass.

The induction of the three germ layer is accompanied with the decreased expression of Oct4 with concomitant induction of expression of germ layer specific marker genes including (1) Otx2 (orthodenticle homologue 2) is expressed in ectodermal cells that give rise to the nervous system and skin. (2)The endoderm is marked by the expression of Hnf4α (hepatic nuclear factor alpha) and Afp (alpha fetoprotein) further differentiating into liver, gut, and lung. Finally, (3) mesodermal cells later forming the heart as well as kidney and skeletal muscles express Brachyury.

The formed mesoderm divides in somatic and splanchnic mesoderm in the anterior part of the embryo during gastrulation (Harvey 2002). Along with Brachyury the splanchnic mesoderm expresses Eomesodermin (Eomes) another T-box transcription factor that induces the transcription of the helix-looped helix transcription factor mesoderm posterior 1 (Mesp1, Costello et al. 2011). Mesp1 induces the expression of early cardiac transcription factors as for example the zinc-finger protein Gata4 and the homeobox protein Nkx2-5 (Bondue and Blanpain 2010). The further specification into cardiomyocytes is depending on signaling cues from the adjacent endoderm like Bmp, Shh, and Fgf signaling proteins (reviewed in Rana et al. 2013). The same holds true *in vitro* where the differentiation of cardiomyocytes is depending on endodermal cells within the cell culture (Liu et al. 2007). To monitor the specification of cardiomyocytes, the expression of α-Actinin, α-Myosin heavy chain, and Myocardin are commonly used. Another protein expressed in the early heart is Alcam which is localized at the cell membrane, such that its detection by e.g. FACS, is possible without applying destructive methods (Murakami et al. 2007). *In vitro*, after the formation of EBs, the cell aggregates are plated onto cell culture dishes where the cells first grow out of the main body of the EB and then differentiate further. Within these regions of outgrowth the formation of cardiomyocytes can be seen by light microscopy in form of spontaneously beating cells (Seiler and Spielmann 2011).

After formation of the tubular heart anlagen the developing heart has to undergo complex morphological changes in order to form the four chambered heart that allows the separation of oxygen-rich arterial from oxygen poor venous blood. Although these morphogenetic transitions can not be seen in a simple ESC 2D differentiation process, the differentiation of ESCs into cardiomyocytes is a very complex, tightly regulated process, in which not only mesodermal cells have to be present but also endodermal and ectodermal cells (Van Vliet et al. 2012). In the next section two of the already mentioned essential signaling cascades governing this process, Bmp and Wnt signaling pathways, will be further elucidated.

1.3 Signaling Cascades

1.3.1 General aspects

In the last section the importance of signaling for developmental processes was introduced, but signaling is a general aspect of multicellular organisms to coordinate cell growth, differentiation, morphology, function, and migration within the organisms but also to react to external stimuli. In general, the signaling cell or introducing cell produces a stimulus which is received and processed by the target cell. The first discrimination is the nature of the signal. Cells can communicate via messenger substances, surface proteins, gap junctions, and electrical signals (Krauss 2014). Since the signaling cascades Bmp and Wnt are

mediated by messenger substances for signal transduction, I will focus on these. The chemical nature of messenger substances is very broad, ranging from small molecules over hormones to proteins by which the signal is transported mediating different ranges of signaling. Accordingly, this is a second aspect for the separation of signaling events (see figure 5). While endocrine signals are transported through the blood and are able to effect distant target cells, most developmental processes are modulated and coordinated by paracrine and autocrine signaling. The effector molecule in paracrine signaling diffuses through the tissue reaching the target cells in a medium distance. Autocrine signaling occurs when producing and target cell are the same cell or of the same cell type (Krauss 2014).

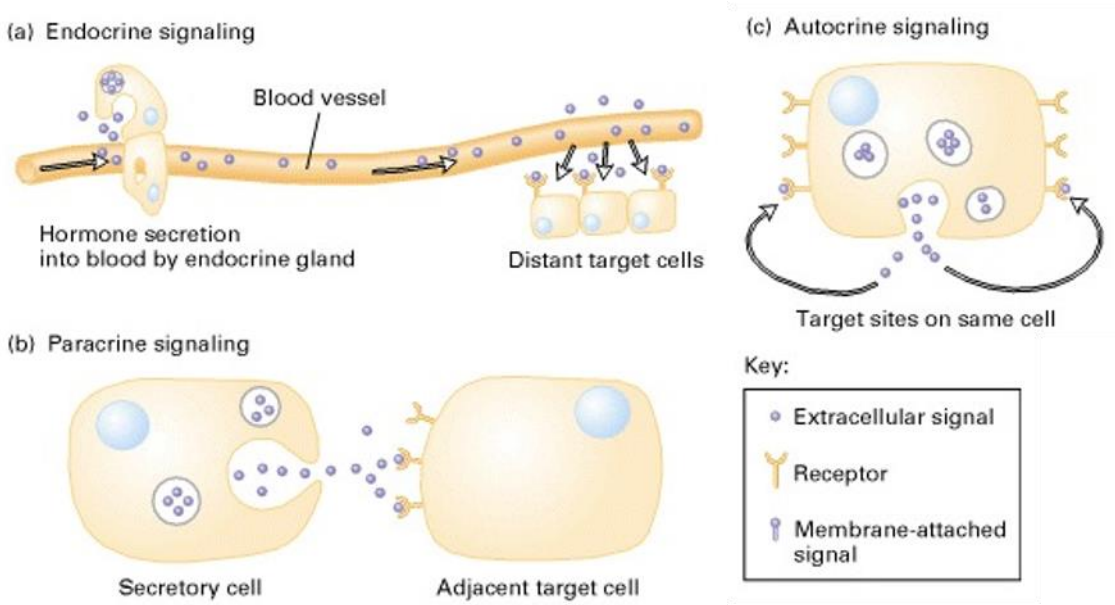

Figure 5: Schematic representation of endocrine (A), paracrine (B), and autocrine (C) signaling. In endocrine signaling the messenger substances (mostly hormones) are transported through blood vessels to reach the distant target cells. Paracrine signaling is of medium range in which the messenger substance (e.g. proteins) diffuse through the tissues to the target cells in close vicinity. Autocrine signaling occurs when the same cell type produces and receives the signal (modified from Lodish et al. 2000).

When analyzing signaling pathways, multiple steps have to be taken into account. The messenger substance has to be produced, modified, possibly stored, and released by the inducing cell (Krauss 2014). These aspects of signaling will not be discussed in this work, although they are very important steps, which as well have to be considered. Then the target cell has to receive the signal and according to its state reacts to the signal. Depending on the type of messenger substance the location of the receptor for the messenger substance can be located within the cell, which is the case for lipophilic hormones that can cross the cell membrane, or receptors on the cell membrane, for signal proteins like Bmp and Wnt (Lodish et al. 2000). Upon binding of the specific receptor the signal has to be processed within the cell, a process

often referred to as signaling cascade, ultimately leading to a cellular reaction. For most of developmental processes this reaction is an alteration of the transcription of target genes thus altering the cell behavior, differentiation status, and with that the cell fate (Loebel et al. 2003). Other reactions can include a change in metabolic activity or the excretion of further messenger substances.

The signal propagation in the cells on a molecular level is an important aspect for the diverse signaling pathways, not only because the reaction of the cell depends on the differentiation status but also on the interpretation of signal strength and length and integration of multiple signals. With increasing knowledge of the molecular mechanisms it is important to stress that the triggered response of the target cells to the same messenger substance can be quite different. Often signaling pathways have one canonical pathway that is the most important and prominent, and sometimes the first elucidated one, and one to several non-canonical pathways, which can have different cellular results and mostly are transduced through a different set of proteins. Nevertheless, the mechanisms by which the signals are transduced within the cells are very similar. One recurring motive of signal transduction within the cell is the phosphorylation of proteins – the addition of a negatively charged phosphate group. Phosphorylations can activate (or inactivate) the target protein, mediated through a change in conformation or generation of binding site for proteins specifically interacting with phoshorylated peptides, as exemplified by the src homology domain 2 (SH2) domain found in various receptor associated proteins that mediates binding to phosphorylated tyrosine. For several signaling cascades, the primary phosphorylated and activated protein is a kinase itself, phosphorylating secondary proteins and, thus, amplifying the primary signal within the cells, as exemplified by the MAPK (mitogen activated protein kinase) signaling cascade stimulated by receptor tyrosine or serine/threonin kinases that amplifies the signaling on several levels (MEKK (MAPKK-Kinase) phosphorylating MEK (MAPK-Kinase) phosphorylating MAPK). Alternatively, the phosphorylated protein can bind secondary proteins and then translocate into the nucleus where it can specifically bind DNA promoters or other transcription factors thereby activating or repressing the transcription of target genes within the cell (Olsen et al. 2006).

The above mentioned discriminations of signaling in general and the discussed mechanisms are only a small excerpt of the plethora of relevant aspects when looking into signaling transduction pathways. Interestingly, there are only a small number of signaling cascades when considering the multitude of tasks for signaling cascades within a multicellular organism in response to extrinsic stimuli or developmental processes. As described in 1.2.2 about five signaling cascades are essential for the differentiation of ESCs into cardiomyocytes and the development of the heart, respectively. The success of the process is dependent on a tide spatiotemporal regulation. Additionally, these signaling pathways are highly

evolutionarily conserved “from fly to men”, indicating that changes in the activity of these signaling pathways in mouse cells upon exposure to teratogenic or toxic substances most likely will also have comparable effects in human cells. In the next two sections, I want to discuss two important signaling pathways, Bmp and Wnt, as examples for such essential, highly conserved signaling cascades (Pires-daSilva and Sommer 2003). For both pathways already existing mouse models were used to generate transgenic reporter ESCs, for which I could establish an *in vitro* test system to analyze the effect of teratogens on these pathways *in vitro*. Therefore, I will explain the main molecular mechanisms and functions as well as describe the transgenic reporter mouse line in each case.

1.3.2 Bmp signaling Pathway

1.3.2.1 Function and molecular mechanisms

In 1965, Urist identified an activity in demineralized bone fractions that induced ectopic bone formation and was therefore named bone morphogenetic proteins (Bmp) (Urist 1965). Subsequently the activity could be purified to an extent that allowed sequence analysis and was found to comprise novel members of Transforming Growth Factor beta (Tgfβ) superfamily, the larges family of growth factors in humans (Sampath and Reddi 1981; Urist et al. 1979; Wozney et al. 1988). By now, the Tgfβ superfamily contains about 30 members that can be subdivided into two main subgroups, the Bmp and the Tgfβ/Activin groups, that have, both, essential and highly conserved functions during vertebrate as well as invertebrate embryogenesis. In particular in gastrulation, early embryonic patterning, and organogenesis but are also involved in tissue homeostasis and repair in adult organisms (Zhao 2003).

Proteins of the Tgfβ family are secreted pre-pro-peptides that can establish an activity gradient within tissues, thereby belonging to the family of paracrine signals. The pre-domain of the Tgfβ protein mediates secretion, whereas the pro-domain (also known as latency-associated peptide, LAP) mediates the interaction of the mature secreted form with components of the extracellular matrix, including Large-Tgfβ-binding proteins (LTBP). Mature Bmp signaling proteins form homodimers as well as heterodimers with other Bmp family members, although the functional relevance and consequence of heterodimerization is still under debate (Constam 2014; Rider and Mulloy 2010). In the extracellular space, Bmp signaling is regulated by additional secreted proteins, including the bona fide Bmp-antagonists Chordin and Noggin (Balemans and Van Hul 2002). These proteins bind to Bmp dimers and prevent the interaction of Bmp with its cognate receptors. For the case of Chordin-Bmp complexes, active Bmp can finally be released upon proteolytic cleavage of Chordin by extracellular metalloproteinases of the Tolloid/Bmp1 family (Zakin et al. 2010). The binding of Bmps to secreted inhibitors and the controlled

release of active Bmp by proteinases are of central importance for the generation of Bmp activity gradients.

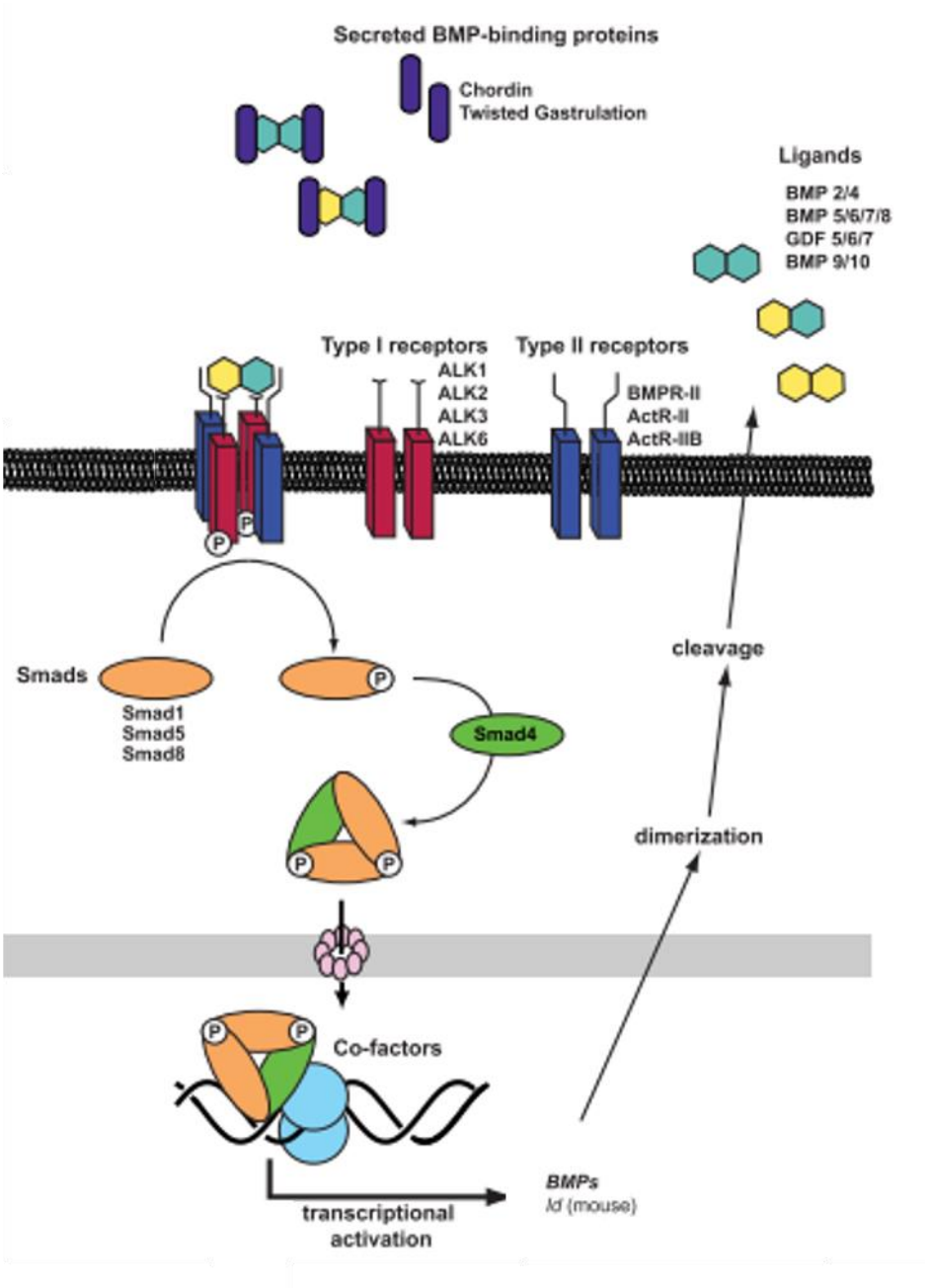

Figure 6: Cartoon depicting the main aspects of the canonical Bmp signaling pathway. The Bmp hetero- and homodimer are bound by secreted Bmp-binding proteins, as for example Chordin. After the release they can bind their receptors, which form heterotetramers, two type I and two type II receptors. After the complex formation, type II receptor phosphorylates type I receptor, which in turn phosphorylates receptor-Smads. Together with the common Smad4, the receptor-Smads form a complex, which shuttles into the nucleus and activate the transcription of target genes together with co-factors after binding Bmp responsive elements (modified from Ramel and Hill 2012).

There are two different types of Bmp receptors belonging to the family of transmembrane serine/threonine kinases. Type I receptors are called anaplastic lymphoma kinase (Alk) 1, 2, 3, and 6 and the most prominent type II receptor is Bmp Receptor-II. Upon binding of the Bmp dimer the type I and type II receptors, which normally reside as homodimer in the cell membrane, form a heteromeric, activated receptor ligand complex. The thus activated receptor type II phosphorylates the type I receptor which in turn phosphorylates and activates intracellular mediators. For Bmp signals, these are the so called receptor Smads, Smad 1, 5, and 8. The activated receptor Smads then bind an additional Smad, common to all Tgfβ signaling, Smad4, and the complex translocates into the nucleus where it binds to specific Bmp response elements (BRE). In contrast, proteins of the Tgfβ/Activin group act through distinct type I and type II receptors that phosphorylate Smad2 and 3 that regulate transcription together with Smad4 via specific Smad binding elements (SBE). In any case, in concert with numerous co-factors, the transcription of target genes is altered, thus mediating a cellular response (see Figure 6; Ramel and Hill 2012).

The above described mechanism is the canonical pathway, which can be regulated and modified extracellularly (as described) and in the cytosol as well as in the nucleus by feedback loops and cross-talk with other signaling pathways. In addition, the activated receptor complex can also activate additional intracellular mediators, including the MAPK pathway, PI3K/Akt and Rho-GTPases, referred to as the non-canonical pathway. These two pathways together form a rather complex intracellular response network (von Bubnoff and Cho 2001; Zhang 2009). Although an enormous amount of research is conducted to further elucidate the exact mechanisms through which the different molecular effects are mediated various aspects are still under debate. Most insights are generated *in vitro,* further complicating the issue since the translation into the *in vivo* situation can be challenging and, thus, needs *in vivo* verification. One possibility to further analyze these mechanisms and validate gathered information are mouse lines harboring an easy detectable reporter gene under the control of Bmp signaling, as for example the BRE mouse line, which I will introduce in the next section.

1.3.2.2 BRE mouse line

In 2008, Monteiro *et al.* published a transgenic mouse line faithfully recapitulating the activity of canonical Bmp signaling through the expression of EGFP, the green fluorescent protein (Monteiro et al. 2008). The gene construct included two BRE sequences together with an enhancer sequence of the cytomegalovirus in front of EGFP. The activity was tested *in vitro* and confirmed the responsiveness of the construct to signals specifically through Bmp receptor type I and demonstrated that reporter activity correlated with increased phosphorylation of Bmp-specific receptor-Smads induced by treatment with Bmp4 *in vitro*. They

generated transgenic mice through pro-nuclear injection of the construct into fertilized oocytes and male offspring showing germline transmission were used to establish the BRE mouse line.

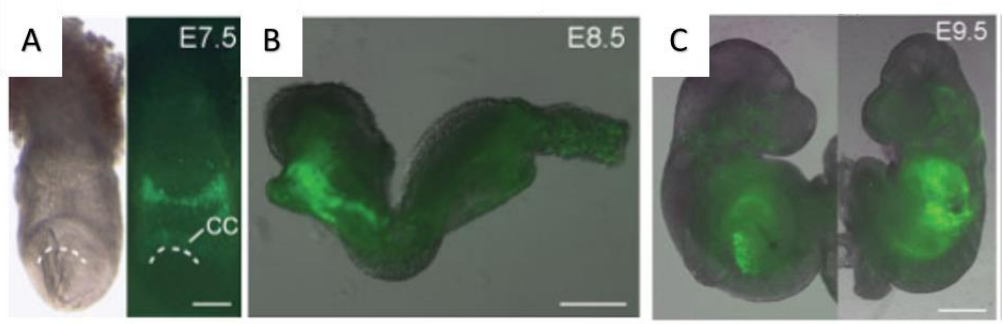

Figure 7: Analysis of EGFP expression during development. (A) At embryonic day 7.5 (E7.5) the EGFP expression can be detected in extraembryonic mesoderm and in the cardiac crescent (CC). At E8.5 (E8.5) staining in the heart anlagen becomes more prominent and increases until E9.5 (C). Later in development, forming blood vessel also express EGFP (modified from Monteiro et al. 2008).

To analyze activity centers of Bmp during development, the expression of EGFP was assessed in mouse embryos at different time points. Very early in development, at embryonic day 7.5 (E7.5), EGFP expression could be detected in extraembryonic mesoderm and the cardiac crescent (see Figure 7A). At E8.5 the expression in the heart anlagen further increases until E9.5 along with additional expression in forming blood vessels (see Figure 7B-C). Later on, Bmp activity can be monitored over the entire embryo, underlining its importance during organogenesis. In figure 8 exemplary organs of all three germ layers at E12.5 are depicted: The heart as mesodermal (8A), the lung as endodermal (8C), and the brain as ectodermal derived organ (8D). In all three cases a broad but distinct EGFP expression was detectable. Additionally, in figure 8B the EGFP in the pre-cartilage and the forming digits can be seen in accordance to the first description of Bmp as a regulator of bone formation. The authors could also show that part of the EGFP positive cells are also positive for PECAM1, a marker for endothelial cells in the lining of blood vessels (compare figure 8E). In this study, the EGFP expression was assessed in embryos or right after birth relying on tissue preparation, although it is possible to detect EGFP also in living animals as has been reviewed by Hoffman (2008). The expression of EGFP correlated nicely with described expression domains of Bmp family members and tissues known to depend on high Bmp activity. Overall, Monteiro *et al.* provided convincing evidence that the BRE mouse line faithfully recapitulates known Bmp signaling centers in the developing embryo rendering it a valuable tool to assess canonical Bmp signaling *in vivo* (Monteiro et al. 2008).

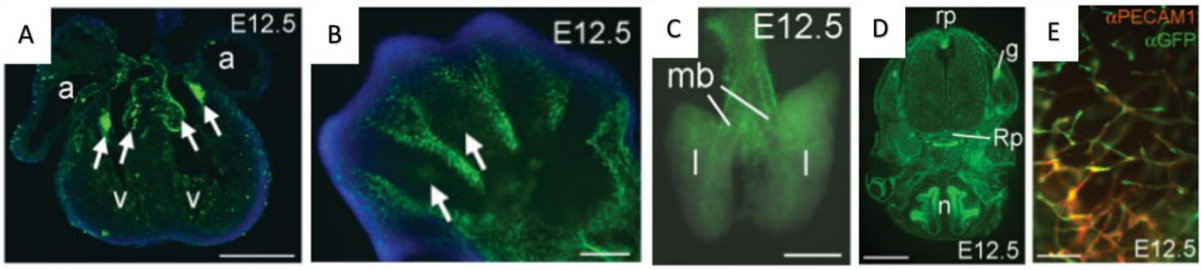

Figure 8: EGFP expression in various organs on embryonic day 12.5 (E12.5). (A) Cross section of the heart. The arrow heads indicate high EGFP expression in the developing atrioventricular valves. (B) Section of the hand plate, in which the arrow heads indicate the forming digits. (C) A picture of developing lungs with the highest EGFP expression in the main bronchi. (D) Transverse section of the brain showing the positive roof plate and Rathke's pouch, later giving rise to the anterior pituitary. (E) Blood vessels of brain tissue with EGFP positive and PECAM1 positive cells. (modified from Monteiro et al. 2008). Abbreviations: a – atrium, g – facial ganglion, l – lungs, mb – main bronchi, n – nasal septum, Rp – Rathke's pouch, rp – roof plate, v – ventricle

1.3.3 Wnt Signaling Pathway

1.3.3.1 Function and molecular mechanisms

The second pathway I focused on during my work was the Wnt signaling pathway. In the 1980s two different proteins exhibiting diverse functions were discovered in mouse and Drosophila melanogaster. *Int-1* was implicated in breast tumors and *wg*, short for wingless, controls segment polarity in drosophila larvae (Nusse and Varmus 1982; Nüsslein-Volhard and Wieschaus 1980). In 1987 it could be shown that these two genes encode homologues proteins leading to the composite name Wnt for proteins of this family (Rijsewijk et al. 1987). This discovery already implicated that Wnt is a highly conserved and essential signaling pathway. In 2012 Clevers and Nusse stated that "The Wnt signal transduction cascade controls myriad biological phenomena throughout development and adult life of all animals" (Clevers and Nusse 2012).

Wnts are acetylated, glycolysated, and lipidated within the endoplasmatic reticulum and these modifications are essential for its activity and secretion (Das et al. 2012). The secretion into the extracellular environment establishes a signaling gradient in a paracrine range. The Wnt/β-Catenin pathway is the canonical transduction cascade involved in cell fate determination and will be explained in more detail below. In contrast the non-canonical Wnt pathways are mediated by specific Wnt receptor subtypes that do not activate β-Catenin but rather stimulate Ca^{2+} release and regulate tissue organization or planar cell polarity instead of cell fate (Yang 2012). Altogether, Wnts can activate various signaling pathways by binding distinct receptors and thereby mediating a large variety of cellular responses (Flaherty et al. 2012; Li et al. 2015).

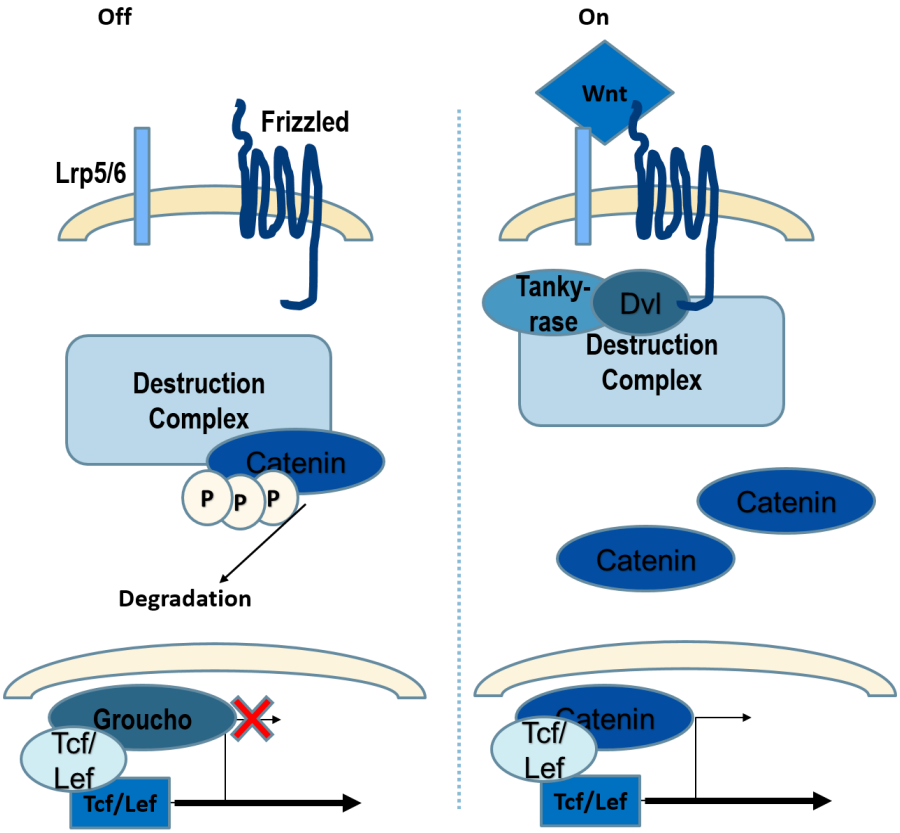

Figure 9: Simplified cartoon of canonical Wnt signaling. During the Off—state β-Catenin is bound by the destruction complex, phosphorylated, and thus targeting it for degradation. Mean while the transcription factors T-cell factor/lymphoid enhancer factor (Tcf/Lef) are bound by the transcription inhibitor Groucho. In the On-State, Wnt binds to its receptors, which with the help of Dishivelled (Dvl) and Tankyrase disassemble the Destruction complex. With that β-Catenin can accumulate and translocate into the nucleus, where it binds to the transcription factors Tcf/Lef thus altering the transcription of its target genes.

The canonical Wnt/β-Catenin signaling pathways has two distinct states, an on- and an off-state. During the off-state, β-Catenin, the intracellular transducer, is bound by the destruction complex. The main components of the destruction complex are adenomatous polyposis coli (APC), Axin, casein kinase 1α (CK1α), glycogen synthase kinase 3β (Gsk3β), and the E3-ubiquitin ligase β-TrCP (Stamos and Weis 2013). Axin is a scaffolding protein mediating the phosphorylation of β-Catenin by CK1α and Gsk3β thereby targeting it for degradation. Especially, Axin and APC are known tumor suppressors proteins and essential for the regulation of Wnt signaling, although the precise molecular mechanisms are still unknown (Stamos and Weis 2013). The extracellular receptors for the Wnt/β-Catenin signaling pathway are a complex of Frizzled, a seven transmembrane protein, and the low density lipoprotein receptor-related protein 5/6 (Lrp5/6). Upon binding of Wnt, the destruction complex is dissolved, the precise mechanisms are not fully understood, but Dishevelled (Dvl) and Tankyrase are two associated proteins, essential for the intracellular transmission of the Wnt signal (Kafka et al. 2014). Through the breakup of the destruction complex, β-

Catenin is not phosphorylated and degraded any more, but can accumulate in the cytosol and translocate into the nucleus. There it binds to transcription factors of the T-cell factor/ lymphoid enhancer factor (Tcf/Lef) family replacing the transcription inhibitor Groucho and, thereby, altering the transcription of its target genes (see figure 9).

The described mechanism of Wnt signal transduction is only a small part of the complex network regulated by components of the Wnt pathway. Most proteins associated are implicated in development and differentiation of cells, as well as described as cause for diseases and cancer when misregulated or mutated (MacDonald et al. 2009). In parallel to the Bmp signaling, the unraveling of the underlying mechanisms requires *in vitro* as well as *in vivo* experiments. Therefore, I want to introduce one possibility in the next section: a transgenic mouse line harboring a reporter gene under the control of Wnt/β-Catenin signaling (**B**eta-Catenin **A**ctivated **T**ranscription, BAT), the BAT-Gal mice that facilitate the *in vivo* analysis of active Wnt/β-Catenin signaling in the developing embryos.

1.3.3.2 The BAT-Gal mice

Already in 2003, Maretto *et al.* published the generation of a transgenic mouse line expressing β-Galactosidase under the control of the β-Catenin regulated *siamois* promoter from Xenopus laevis (Maretto et al. 2003). In their study, Maretto *et al.* focused on Wnt/β-Catenin activity during mouse development and in cancer. The β-Galactosidase protein is originally found in bacteria and induced by high lactose levels to facilitates the cleavage of the β-glycoside bond rendering the bacteria able to use lactose as an alternative food source (Jacob and Monod 1961). The drawback of this reporter gene lies in the fact that it can only be detected indirectly. In order to detect β-Galactosidase activity, an artificial substrate like X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) has to be applied which will be cleaved under formation of a blue colored reaction product that can be monitored and used as an indicator for the reporter gene expression.

First the specificity of their gene construct was demonstrated by specific induction through active Wnt/β-Catenin signaling in cell culture. The gene construct was then introduced by pronuclear injection in fertilized oocytes and a transgenic mouse line was established. In the early embryo Wnt/β-Catenin activity centers were mainly detected in the primitive streak and the forming mesoderm. Later on, the transgene was also detected in the mid- and hindbrain, forming somites, as well as the developing heart (compare figure 10). During organogenesis active Wnt/β-Catenin signaling was seen in the kidney, lung, trachea, and bronchi. Additionally, the authors could detect the reporter gene in neural crest cells migrating through the embryo after epithelial to mesenchymal transition (Maretto et al. 2003). With that the authors could show that the reporter gene construct faithfully recapitulates known Wnt/β-Catenin activity center and

that the transgenic mouse line might help to dissect the role of Wnt signaling during embryogenesis *in vivo*.

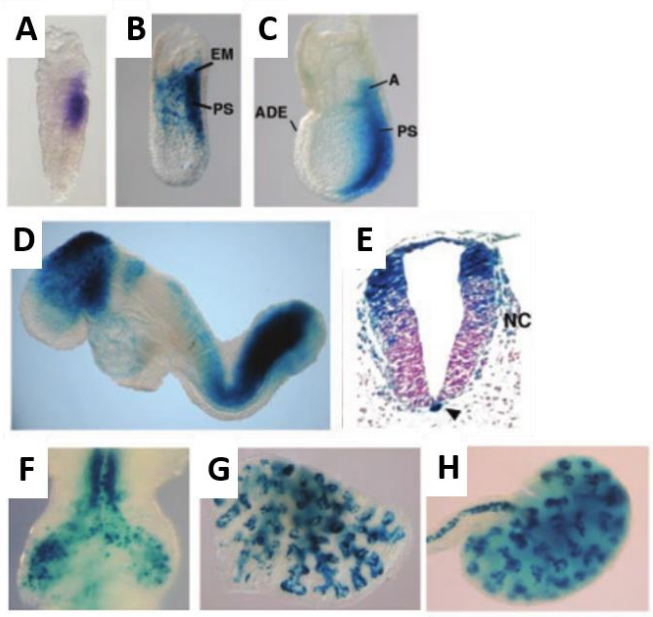

Figure 10: X-gal *in situ* staining of β-Galactosidase of early embryonic stages of the BAT-Gal mouse line. (A) At embryonic day 6.0 (E6.0) the blue staining can be seen in the posterior site at the junction between embryonic and extraembryonic ectoderm and intensifies during gastrulation in the primitive streak (PS, B and C). (D) At E8.5 the transgene can be detected in the developing heart, mid- and hindbrain regions, and the developing somites. (G) The cross section of the neural cord reveals that neural crest cells (NC) are also positive for the reporter gene. The arrow head indicates the notochord. Wnt/β-Catenin signaling is also important during organogenesis, as can be seen in the trachea and bronchi (F), the lung (G), and the kidney (H, modified from Maretto et al. 2003).

1.4 Substances

The realization that environmental factors can have an impact on the developing embryo was laid in 1941, when Gregg could associate measles with congenital disorders (Gregg 1941). With the contergan scandal in the early 1960s, the need to assess the teratogenic potential of substances became even more evident. In 1965, Karnofsky reviewed the knowledge of its time, thereby concluding that a teratogenic potential of a given substance can only be determined in relation to the toxic effect on the mother, in particular a true teratogenic effect should be detectable without any obvious maternal toxicity (Karnofsky et al. 1965). For validation of *in vitro* alternatives to animal tests, Brown classified the substances roughly into three different groups according to their teratogenic potential, strong, weak, and non-teratogenic substances (Brown 2002). Non-teratogenic substances show no evidence of having an adverse effect on the developing embryo, whereas strong teratogens exhibit clear adverse effects in all species tested. The

intermediate group, termed weak teratogens, does not display a teratogenic effect in all tested species or lack relevant human data. Additionally, in some cases the medical indication and benefit might outweigh the potential risk. It should be kept in mind that following this classification system, thalidomide/contergan would be called a weak teratogen, despite the severe effects it can cause in humans.

Establishing an *in vitro* test system requires the determination of its predictivity. Brown described the logic in the selection of test substances (Brown 2002): (1) Only directly active substances should be used, because most *in vitro* test systems lack the necessary metabolic activity, although this issue might be overcome with conditioned media. (2) He excluded endocrine-active substances since it is a specific mode-of-action (MoA) for which *in vitro* test systems are already available. (3) The used substances should exhibit different molecular mechanisms to allow the evaluation of MoA dependent predictivity and elucidate strength and weaknesses of the test system further. (4) In order to be able to evaluate the *in vitro* data sufficient *in vivo* data has to be available and (5) if possible also human data. For my studies I used substances that were selected for in the validation of the embryonic stem cell test (EST) to have sufficient reference data to compare my results with. In the next sections I will shortly describe their use and MoA as far as they are known.

1.4.1 Non-teratogenic substances

The choice of non-teratogenic substances as negative controls is of particular importance. In the case of my test system, the substances should exhibit an unspecific cellular toxicity at high concentrations without having an effect on the analyzed signaling pathways or cardiomyocyte differentiation at lower concentrations.

The first substance selected was diphenhydramine hydrochloride (DPH). DPH is a first-generation antihistamine that was first synthesized in 1941 and approved by the FDA in 1946, known under the trademark Benadryl. It is used to treat allergic reactions, insomnia, nausea from motion sickness, and in the US also to treat movement disorder symptoms of, as for example, Parkinson's disease (Casterline and Evans 1977; Kudo and Kurihara 1990; McGeer 1961). The teratogenic potency of DPH was assessed in rats and rabbits where no congenital disorders could be found, although the rabbits of the high dose group showed weight gain depression (Schardein et al. 1971). A retrospective analysis of antihistamine treatment during pregnancy could not find any adverse effects of DPH (Li et al. 2013).

The second control substance was Acrylamide, a commonly used chemical in molecular biology and used as a reagent to create a matrix for the separation of for example proteins (Polyacrylamide Gel Electrophoresis, PAGE). Through a radical reaction, polymers can easily be produced that account for the

toxic effects for acrylamide within cells. A second source for acrylamide is the heat dependent Maillard-reaction between amino acids (like asparagine) and reducing sugars in carbohydrate-rich food, including french fries or potato chips (Keramat et al. 2011). Acrylamide is considered to be a neurotoxic and carcinogenic compound, and although it was found to exhibit reproductive toxicity in animal studies no embryotoxic or teratogenic effect in humans could be observed (Exon 2006) and it was classified as non-teratogenic substance (Brown 2002).

1.4.2 Teratogenic substances

As teratogenic model substances I have chosen 6-aminonicotinamide, valproic acid, retinoic acid, and lithium chloride.

Retinoic acid (RA) is defined as strong teratogen and is used to treat acute leukemia and acne (Gollnick and Schramm 1998; Warrell Jr et al. 1991). It is a metabolite of vitamin A and a naturally occurring morphogen and signaling molecule, essential during embryogenesis and in adult tissue homeostasis. RA is a lipophilic molecule able to cross the cell membrane and to shuttle directly into the nucleus where it binds its receptors: retinoic acid receptors (RAR) and retinoid X receptors (RXR). These form heterodimers and bind to RA responsive elements to alter the expression of target genes. During development it is essential for the correct patterning of the embryo and brain development (Rhinn and Dolle 2012). With that the teratogenic potential of excess, exogenous RA during embryonic development is thoroughly analyzed and well understood (Horton and Maden 1995; Mark et al. 2006).

6-Aminonicotinamide (ANA) is classified as a strong teratogen and a potent nicotinamide antagonist (Johnson and McColl 1955). Thus, ANA inhibits Glucose-6-phosphate dehydrogenase which is an important enzyme of the pentose phosphate pathway (PPP, Herken and Lange 1969). The PPP is a metabolic pathway generating ribose-5-phosphate, pentoses, and NADPH, thereby producing substrates and reductive agents for biomolecule synthesis, like nucleotides and aromatic amino acids. Its function already implies how ANA induces its teratogenic effects. Additionally, the PPP is closely associated with glycolysis, the anaerobic pathway to generate energy from glucose and the main energy producing pathway in cancer cells instead of the aerobic citrate cycle. This metabolic change is called Warburg effect and one approach for cancer therapeutics is based on the interference with the PPP as a key component of cancer cell metabolism and survival. In this light, ANA is as well analyzed and actually approved by the US Food and Drug Administration (FDA), mostly in combination with other chemicals to block glycolysis or to induce radio sensitivity in tumors (Chakrabarti et al. 2015; Pelicano et al. 2006), although the side effects elicited by ANA are severe (Herter et al. 1961).

Valproic acid (VPA) is an anticonvulsant and mood stabilizer and classified as a weak teratogen. In several animal studies VPA induced congenital abnormalities, like spina bifida (Kao et al. 1981). In human the long term treatment with VPA increases the risk of congenital malformations three fold and causes facial abnormalities, termed the valproate syndrome (Ornoy 2009). The mechanism by which the effects of VPA are induced are still under investigation, but one of the main aspects seems to be the inhibition of histone deacetylases (HDACs), which alter the acetylation status of histones thereby changing the availability of genes for transcriptional activation, regulating cell cycle, and development. In addition VPA has been described as inducing β-Catenin accumulation and β-Catenin dependent transcription partially mimicking active Wnt signaling (Wiltse 2005).

The last weak teratogen I used as a model substance is lithium chloride (LiCl), which is also a mood stabilizer. LiCl inhibits inositol monophosphatase (IMPase) as well as Gsk3β thereby stimulating, similar to VPA, canonical Wnt signaling. However, its neuroprotective and teratogenic effects seem mainly be mediated by the inhibition of Gsk3β (Wexler et al. 2008). LiCl is not associated with a higher risk of adverse effects in therapeutical doses, although teratogenic effects have been reported (Aral and Vecchio-Sadus 2008).

The six chemicals that were chosen for the characterization of the test systems have very distinct mechanisms and the available data increase the possibility to understand and discuss our findings and with that the predictivity and applicability can be assessed and evaluated.

2. Objective

Teratogenic effects of substances are mainly assessed in animals. And although first alternatives were validated, a regulatory acceptance has not been achieved so far. Especially for the EST, a main point of criticism was that only differentiation into cardiomyocytes is assessed. Our approach was to analyze whether ESCs with easily detectable reporter genes under the control of essential signaling cascades can be used to predict teratogenic activities. The species difference in the reaction to exogenous substances complicates the prediction of teratogenic activities. Therefore we concentrated on highly conserved signaling pathways, as Bmp and Wnt signaling. As test system the differentiation of ESC into cardiomyocytes was used, for one because the EST itself performed very well in validation studies and the analysis of the signaling pathways was thought to give further mechanistic information on the mode of action of the teratogenic compounds. Further, the differentiation into cardiomyocytes *in vitro* can be seen spontaneously, without additional factors that might interact with the tested substance thereby compromising the results. And even more important, the differentiation into functional cardiomyocytes depends on the simultaneous formation of the other two germlines broadening the possible mode of actions to interfere with this readout

I was able to work with two different transgenic mouse embryonic stem cell lines which were generated in the laboratory of Prof. R. Kemler (MPI-IB, Freiburg). These cell lines were derived of the BRE and the BAT-Gal transgenic mouse lines, respectively (compare sections 1.3.2.2 and 1.3.3.2). Following aspects were addressed in order to establish the *in vitro* test systems:

1. The pluripotency of the cell lines had to be ascertained and the differentiation process into cardiomyocytes characterized and compared to the literature.
2. Qualitative and quantitative analysis methods needed to be established to measure and characterize the reporter gene activity during the differentiation process.
3. Although each construct had been validated *in vivo*, its specificity *in vitro* using specific and direct inhibitors of the pathway had to be verified.
4. To further verify the biological relevance of reporter gene expression and to provide additional information on the mode of action of test substances the identity of reporter gene positive cells was analyzed.
5. An easy to perform and suitable method for the identification of teratogenic compounds based on effects on the reporter gene expression had to be established.

In the following section, the results of my work are presented.

3. Results

3.1 A Bmp reporter transgene mouse embryonic stem cell model as a tool to identify and characterize chemical teratogens

First I want to present the results of the ESC generated from the BRE mouse line: the BRE-ESC. The Bmp signaling pathway is essential for the correct patterning of the embryo and acts on the cardiac crescent *in vivo*. Later the influence of Bmp signaling intensives during functional specification of cardiomyocytes *in vivo* and *in vitro*. The BRE mouse line faithfully recapitulated these findings and I could confirm this in the test system.

I was indeed able to establish them as tool to predict teratogenic effects *in vitro*. First, I established the protocols to quantitatively measure the reporter gene expression on the protein level by quantification of fluorescence as well as on the transcriptional level by quantitative PCR. In combination with the analysis of marker genes, the success of the differentiation could be monitored and Bmp activity could be correlated with specific phases of differentiation, e.g. mesoderm induction. Additionally, we could verify the specificity of the reporter gene using LDN193189, a specific inhibitor blocking the intracellular transmission of the Bmp signal that effectively and specifically inhibited the expression of the EGFP reporter in a time dependent manner. In addition, LDN193189 prevented the formation of endoderm, an essential tissue for the specification of functional cardiomyocytes. I could show that the EGFP positive cells are endothelial and endodermal cells. Using model substances for teratogenicity, I was also able to show that the quantification of EGFP expression as well as the morphology of EGFP positive cells are suitable readouts for the identification of teratogenic activity, in particular since effects on EGFP expression were detected at concentrations that were comparable to the ones interfering with cardiac differentiation.

The author's contribution:

Design of experimental approach, conduction of experiments, evaluation, and interpretation of experimental data

Preparation of manuscript and figures

A Bmp reporter Transgene Mouse Embryonic Stem Cell Model as a Tool to Identify and Characterize Chemical Teratogens

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Toxicological Sciences 2015 Aug;146(2):374-85

doi: 10.1093/toxsci/kfv103

A Bmp reporter transgene mouse embryonic stem cell model as a tool to identify and characterize chemical teratogens

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Abstract

Embryonic stem cells (ESCs) were first isolated from mouse embryos over 30 years ago. They have proven invaluable not only in generating genetically modified mice that allow for analysis of gene function in tissue development and homeostasis but also as models for genetic disease. In addition, ESCs *in vitro* are finding inroads in pharmaceutical and toxicological testing, including the identification of teratogenic compounds. Here we describe the use of a bone morphogenetic protein (Bmp)-reporter ESC line, isolated from a well-characterized transgenic mouse line, as a new tool for the identification of chemical teratogens. The Bmp-mediated expression of the green fluorescent protein enabled the quantification of dose- and time-dependent effects of valproic acid as well as retinoic acid. Significant effects were detectable at concentrations that were comparable to the ones observed in the classical embryonic stem cell test (EST), despite the fact that the reporter gene is expressed in distinct cell types, including endothelial and endodermal cells. Thus these cells provide a valuable new tool for the identification and characterization of relevant mechanisms of embryonic toxicity.

Key words: BMP, differentiation, stem cells, GFP, reportergene, embryo

Introduction

Multiple dynamic processes regulate cell differentiation and organ morphogenesis during embryonic development. How these complex processes are affected by toxic reagents is still largely unknown. The assessment of chemicals that might pose a serious hazard to human (developmental) health therefore still mainly relies on the use of experimental animals. Recent changes in legislation restricting the use of experimental animals, in particular in Europe, has initiated research into alternative approaches to address developmental toxicity by taking advantage of the underlying molecular mechanisms (Whelan and Andersen, 2013). Two recent contributions to this kind of research include: (i) improvements in bioinformatics and analytics that allow comprehensive spatial-temporal analysis of cellular responses on the molecular level (Sturla *et al*., 2014); and (ii) new cell and embryo culture techniques that provide relevant mechanistic information (Sipes *et al*., 2011). Nevertheless, a comprehensive and predictive testing strategy for embryotoxicity is not anticipated in the near future (Adler *et al*., 2011).

Another trend in understanding developmental processes utilizes genetically modified animals and derived cell lines thereof. Such systems have the advantage that direct comparison of *in vitro* and *in vivo* data is possible and the influence of chromosomal integration sites on the biology are either known or can be excluded (Wilson *et al*., 1990). Signaling pathways modulated by toxic reagents are often in the focus of studies using such cell lines for risk assessment and mechanistic insights (Boverhof *et al*., 2011). The first steps in embryogenesis—including germ layer formation during gastrulation, axis specification, proliferation, differentiation, cell death, and cell migration—are controlled by rather few highly conserved signaling pathways (Loebel *et al*., 2003). Among these the bone morphogenetic protein (Bmp) signaling pathway is of particular importance. This pathway, as part of the transforming growth factor (Tgf) β signaling pathway, is evolutionarily highly conserved both in structure and function, and is crucial during embryonic development in many vertebrate and invertebrate species (Bier, 2011). The Bmp protein family consists of over 20 secreted molecules in mammals that induce the assembly of heteromeric receptor complexes of type I and type II transmembrane serine/threonine kinases. The type II receptor kinase activates the type I receptor kinase, which subsequently phosphorylates receptor-Smad proteins (R-Smads, Smad 1, 5, 8) at its C-terminus. This phosphorylation then stimulates the binding of R-Smad to a common cofactor, Smad 4, leading to the translocation of the complex into the nucleus. The transcriptional regulation of target genes is facilitated through the binding of the complex to Bmp response elements (BRE) (reviewed in Weiss and Attisano, 2013).

Here we describe a new embryonic stem cell (ESC) line that has been isolated from a transgenic mouse line carrying a BRE promoter (BRE-ESC) upstream of an enhanced green fluorescent protein (EGFP). *In vivo*,

EGFP expression was detected in the extra-embryonic mesoderm at day 7.5, followed by expression in the developing heart, blood vessels as well as in kidney, bone, liver, pancreas, lung and hair follicles at later stages. Although some known Bmp activity centers did not express EGFP, the overall reporter gene activity correlated well with known biological activities of Bmp signals (Monteiro *et al*., 2008). The ESCs derived from these mice could then in principle report Bmp signaling faithfully in culture at different stages of differentiation using the standard procedure developed during the validation of the embryonic stem cell test (EST, Seiler and Spielmann, 2011). The dependence of differentiation on Bmp signaling made the BRE-ESCs a possibly suitable tool to assess inhibitory effects of substances and might broaden the predictivity of the EST. Our results indicate that the *in vitro* reporter gene activity at least partially recapitulates the *in vivo* expression pattern and might serve as a predictive marker for teratogenic activity, providing a new tool for the identification of potentially teratogenic substances.

Material and Methods

ESC culture

The BRE-ESCs were derived from the transgenic reporter mice as previously described (Doetschmann *et al*., 1985). Experiments were performed in agreement with the German law on the use of laboratory animals as well as biosafety (S1) and institutional guidelines of the Max Planck society. The use of animals was approved by "Regierungspräsidium Freiburg (Freiburg regional council)" and the animal welfare office of the Max Planck Institue of Immunobiology and Epigenetics, Freiburg, Germany (KE-2iTO-6). BRE-ESCs were routinely passaged every two to 3 days as described elsewhere (Seiler and Spielmann, 2011) with minor changes. The culture media contained high glucose (4.5 g glucose/l) DMEM (Gibco Life Technologies, Karlsruhe, Germany), 15% fetal calf serum (Gibco), 2mM glutamine (Gibco), antibiotics (50 U/ml penicillin, and 50 µg/ml streptomycin; Gibco), 1% nonessential amino acids (Gibco) and 0.1 mM β-mercaptoethanol (Sigma, Deisenhofen, Germany). During the routine maintenance passage the cells were kept in 1 µM PD0325901, 3 µM CHIR99021 media and 1000 U/ml murine leukemia inhibitory factor (mLIF; Chemicon, Hofheim, Germany) (Ying *et al*., 2008).

Differentiation protocol

Hanging drop culture was performed as previously described (Seiler and Spielmann, 2011). In brief, a cell suspension of $0{,}5x10^5$ cells/ml was prepared in culture media without LIF, CHIR99021 and PD0325901. 20 µl drops were made on the lid of a 10 cm cell culture dish and incubated for 3 days at 37°C and 5% CO_2. After 3 days, EBs were transferred into suspension culture for 2 days and then cultivated on cell culture dishes for 2 to 7 days. For subsequent analysis of differentiation into cardiomyocytes single EBs were

transferred into 24-well plates on day 5, for qPCR, EGFP-Assay, and FACS analysis 25 EBs each were cultured on 6-well plates from day 5 onwards.

qPCR

Total RNA was extracted using Trizol (Life Technologies, USA) according to the supplier information. Total RNA extracts were resuspended in 45 µl water and subsequently 5 µl 10x DNase Buffer and 1 µl DNase (NEB, England) were added. After 30min at 37°C, the reaction was stopped on ice, purified using phenol/chloroform (Roth, Germany) and precipitated with 100% ethanol and sodium acetate (3M). cDNA was generated from 2 µg total RNA using HighCapacity cDNA Reverse Transcription Kit (Life Technologies, USA) according to the information provided by the manufacture. cDNAs were diluted 1:10 in water and 1 µl was used for each qPCR reaction on a 7500 Fast Real-Time PCR System (Life Technologies, USA). Gene expression were standardized against Gapdh and normalized against the appropriate control. Standard deviation (SD) was calculated from triplicates with differences > 3xSD were considered statistically significant.

Table1: List of primers used in qPCR analyses of the indicated genes.

Gene	Forward	Reverse
Gapdh	AGTGCCAGCCTCGTCCCGTA	CAGGCGCCCAATACGGCCAA
Alcam	CAGTGGGAGCGTCATAAACC	CCTCAGGGGAAATGATAATTTACTA
Gata4	GGAAGACACCCCAATCTCG	CATGGCCCCACAATTGAC
Brachyury	ATGCTGCAGTCCCATGATAAC	TGCGTCAGTGGTGTGTAATGT
CD31	GTCATGGCCATGGTCGAGTA	CTCCTCGGCATCTTGCTGAA
Flk1	TCTGGACTCTCCCTGCCTAC	TGATGCAAGGACCATCCCAC
αMhc	CGCATCAAGGAGCTCACC	CCTGCAGCCGCATTAAGT
Hnf4α	GCAAGGGGTTCTTCAGGAGG	TGCTGTCCTCGTAGCTTGAC
Afp	GGCCGACATTTTCATTGGACAT	TGGGGGAGGGGCATAGGTTTT
Oct4	GGAGGGATGGCATACTGTGGACCT	AGCTCCTTCTGCAGGGCTTTCA
EGFP	TTCAGCCGCTACCCCGACCA	GCCCCAGGATGTTGCCGTCC
Id1	GGCGAGATCAGTGCCTTG	AAGGGCTGGAGTCCATCTG
Smad6	CATCACTGCTCCGGGTGAAT	GCCCTGAGGTAGGTCGTAGA
Map2	GGTTCCAAGGATAACATCAAACA	CATTTGGATGTCACATGGCTTA

Western Blot

Samples were taken at indicated time points. Cells were lysed in lysis buffer [20 mM Tris, 138 mM NaCl, 5 % glycerin, 4 mM EDTA, 1 % Triton X-100, 5 mM β-mercaptoethanol supplemented with 1 x protease inhibitor mix (Roche, Germany) and 0,1 mM sodium orthovanadate] for 10 min. After centrifugation, the protein concentration was determined using the BCA assay (ThermoScientific, USA) according to manufacturer's protocol. 40 µg of protein lysate were separated on a 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred on a nitrocellulose membrane (BioRad, USA), after blocking the membrane for 1 h in 5 % milk powder in TBST, the primary antibody incubation was performed in blocking solution over night at 4°C. The antibodies were diluted at 1:500 for anti-Oct4 (sc-9001), anti-Gata4 (sc-9053) and anti-Brachyury (sc-17743). Anti-EGFP (ab13970; Abcam, England), anti-Alcam (AF1172, R&D Systems, USA) and anti-Tubulin (Abcam, England) were diluted 1:2000, anti-pSmad1 (D40B7, Cell signaling, USA) 1:1000. The appropriate secondary antibody coupled to horseradish peroxidase (Dianova, Hamburg) was diluted 1:10000, incubated for 1 h at room temperature and detected with SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific, USA) with a ChemiDocs XRS (BioRad, USA).

EGFP-Assay

For the quantitative measurement of EGFP, at least 25 EBs were trypsinized for 15 min at 37°C, rinsed and pooled. After centrifugation the pellet was lysed with 60 µl M-PER Mammalian Protein extraction Reagent (ThermoScientific, USA) for 2 h at room temperature or overnight at 4°C. The lysates was centrifuged to remove cell debris and 50 µl of the lysate were transferred into a 96-well plate. The EGFP amount was measured with a Tecan Infinite M2000 plate reader (Tecan Group Ltd., Switzerland) with a excitation wavelength of 485 nm and a emission wavelength of 525 nm. Subsequently, the protein amount in the lysate was determined with a Nano-Drop 1000 (PeqLab Biotechnology GMBH, Germany) using the absorption at 280 nm. After substracting the blank values, the EGFP values were standardized against the protein amount and normalized against the appropriate control. Standard deviation (SD) was calculated from triplicates with differences > 3xSD were considered statistically significant. .

The Cell Titer Blue assay (Promega, USA) was performed as described in the protocol provided by the manufacturer. 1ml of CTB in media (1:10) was used per 6-well for 1h. Triplicates were sampled (100 µl) and measured with a Tecan Infinite M2000 plate reader, excitation wavelength 560 nm and emission wavelength 590 nm.

FACS

EBs were incubated with TrypLE™ Express (Life Technologies) and EDTA (PAN, Germany) for 15 min at 37°C. Afterwards a single cell suspension was created by vigorously pipetting the cell suspension with FACS buffer (PBS with 5 % FCS and 1 % EDTA) and filtered through falcon tubes with cell strainer caps (Fisher Scientific). Cell suspension was stained using the Live/Dead fixable near IR staining Kit (1:1000, Life Technologies) and anti-CD31-BV421 (1:100, 390, BioLegend, USA) for 30 min at room temperature. The samples were fixed with CellFix (BD Biosciences, USA) and stored at 4°C until FACS analysis with a FACS Aria III (BD Biosciences, USA). The evaluation was performed using FlowJo (Tree Star, USA).

Immunofluorescence

For immunofluorescence studies, EBs were seeded on cover dishes. On day 10 or day 12, DiI-Ac-LDL was applied to the EBs for 4 h at 37°C and 5% CO_2. Then, EBs were treated with 4 % paraformaldehyde for 20 min and 0.5 % Triton-X-100 for 10 min. Primary antibodies were incubated for 90 min at room temperature: CD31 (1:400, MEK13.3, BD Biosciences), Flk1 (1:400, sc-6251, Santa Cruz) and MF20 (1:500, Developmental Studies Hybridoma Bank, USA). The appropriate secondary antibodies coupled to Alexa Fluor 546 (Life Technologies) were diluted 1:200 and incubated for 30 min. DAPI staining was performed for 5 min.

Results

Characterization of differentiation of BRE-ESCs

For efficient differentiation we used a standard procedure developed in the frame of the validation of the embryonic stem cell test (EST, Seiler and Spielmann, 2011). Applying this protocol, we tested the differentiation potential of the BRE-ESCs and analyzed changes in EGFP expression by fluorescence microscopy (Fig. 1A). Low levels of EGFP expression were already detectable in BRE-ESCs before induction of differentiation, indicating some Bmp signaling activity in the pluripotent ESC culture. After 4 days of embryoid body (EB) formation, the expression of EGFP decreased with some cells at the outer layers of EBs still expressing EGFP. Upon stimulation of EB outgrowth, and subsequent to plating the EBs on cell culture dishes, the number of cells expressing EGFP increased, with more EGFP-positive cells detectable in the leading edge of the EB outgrowth (Fig. 1A, d6 – d12). During the subsequent days of differentiation, EGFP expression continued to increase and morphologically distinct EGFP-positive cell populations became detectable (Fig. 1A, d10 and d12). At day 10 of differentiation, some EGFP-positive cells were associated with beating cardiomyocytes without displaying any contracting activity themselves. Another distinct EGFP-positive cell population developed into net-like structures that became visible at day 10 to

12 of differentiation. To verify the differentiation potential and to analyze the differentiation kinetics of the BRE-ESCs in more detail, we performed qPCR and Western blot analysis of marker genes for pluripotency (Oct4), mesoderm induction (Brachyury) and cardiomyocyte differentiation (Gata4, Alcam, alpha Myosin heavy chain (αMhc)) (Fig. 1B, C). As expected Oct4 protein as well as transcript levels decreased during the differentiation process. Brachyury, a marker for pan-mesodermal cells, became detectable on the protein as well as transcript level on day 5 of differentiation. The initial decrease in the Brachyury transcription expression level is probably due to the fact that the BRE-ESCs were kept under so-called 2i (two inhibitor) conditions (Ying *et al*., 2008) during routine cell culture. Brachyury is a well-known Wnt target gene and the 2i media includes the Gsk3β inhibitor CHIR99021 that mimics Wnt signaling activity (Arnold *et al*., 2000; Bain *et al*., 2007). The transcription of the mesodermal markers concurred with mesoderm induction although the protein levels displayed the expected delay. Gata4, a marker for cardiomyocyte precursors, is also transcriptionally induced between day 3 and day 5 of differentiation, but the protein can first be detected on day 7. Similarly, the expression of αMhc is induced from day 7 onward and Alcam protein, as marker for the heart region in early embryos (Murakami *et al*., 2007), could not be detected before day 10. Thus, the marker genes indicate the proper differentiation of the EBs. In addition, we analyzed the expression of the direct Bmp target genes Id1, Id3 and Smad6 as well as the phosphorylation status of the Bmp signaling mediator Smad1. The qPCR analysis of the expression of Bmp marker genes indicated an induction of endogenous Bmp signaling activity at day 5 (Fig. 1D). EGFP protein itself could also be detected in undifferentiated BRE-ESCs and was subsequently induced from day 5 onward. Low Smad1 phosphorylation could be detected already in undifferentiated BRE-ESCs. It was not detected at day 3 and 5 but was induced again from day 7 onward (Fig. 1B). The somewhat delayed detection of proteins, in particular of the phosphorylated Smad1, might be due to lower sensitivity of Western blots that is also highly dependent on the respective antibody. Additionally, the phosphorylation of Smad1 represented the actual signaling status of the cells, whereas the expressed EGFP as well as the expression of Brachyury documented the activity of Bmp signaling over a longer time period. Altogether, EGFP expression nicely correlated with the transcriptional levels of Bmp target genes and, in particular in respect to the biphasic Bmp activity profile, with a consistent phosphorylation status of Smad1, confirming that EGFP expression indeed indicates endogenous Bmp signaling activity as had already been shown *in vivo*.

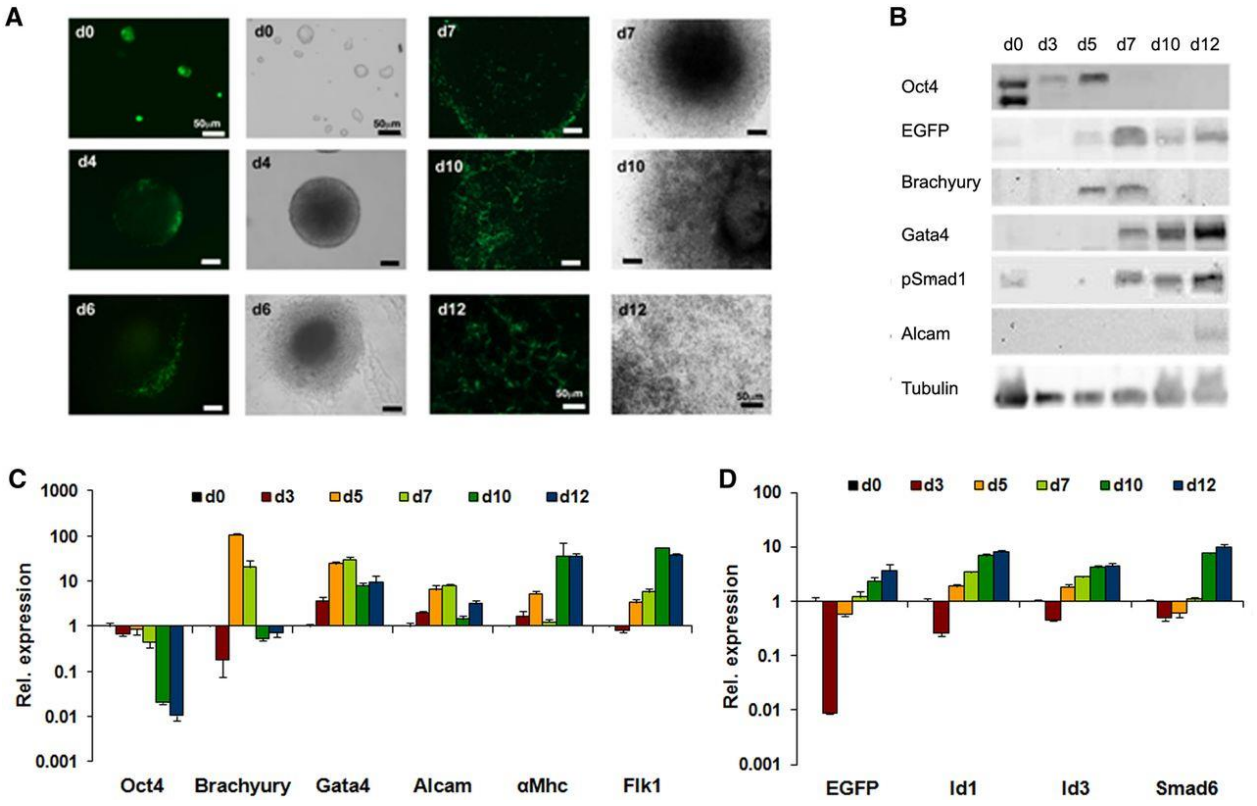


Figure 1: Marker gene expression in BRE-ESCs during differentiation. (A) EGFP expression of BRE-ESCs during differentiation by the hanging drop method. Brightfield and fluorescence pictures taken with an Apotome2 microscope of the same ESC culture are shown for each time point. The scale bars indicate 100 µm, except for day 0 and day 12 (50 µm). (B) Western blot analysis of the indicated marker genes for pluripotency (Oct4), mesoderm induction (Brachyury/ T), mesoderm differentiation (Gata4, Alcam), Bmp activity (phospho-Smad1) and EGFP at the depicted days of differentiation. α-Tubulin served as an internal loading control. (C) qPCR analysis for Oct4 (pluripotency), Brachyury (mesoderm induction), Gata4 (early mesoderm), Alcam, αMhc (heart) und Flk1 (endothelial cells) at the indicated days of differentiation. (D) qPCR analysis of EGFP and Bmp target genes (Id1, Id3, Smad6) during differentiation. For qPCR, the samples were standardized against Gapdh and the expression levels normalized against undifferentiated ESC.

Quantification of EGFP expression

To quantify the EGFP expression over the course of differentiation, we tested whether the EGFP expression can be measured directly in cell lysates. To ensure sufficient EGFP expression, 25 EBs from day 7 of differentiation were pooled for each measurement and the EGFP expression was quantified using a Tecan Infinite M2000 plate reader (excitation: 485 nm, emission: 535 nm). In a serial dilution experiment the relative EGFP amount nicely correlated with the relative protein amount (Fig. 2A), thereby allowing a linear slop regression (R^2=0.9968). In addition, different methods for standardization were tested. Figure 2B shows the correlation between cell viability, determined by CTB measurements and the protein amount within the lysate using the absorption at 280nm (OD_{280}). Since there were no significant differences

applying either of the two methods, standardization was further carried out using the protein amount determined via OD_{280} in all subsequent experiments. In following experiments, the cultures were evaluated microscopically before lysate preparation and lysates that displayed a clear reduction of the protein concentration indicating unspecific, general toxic effects, were excluded from further analysis.

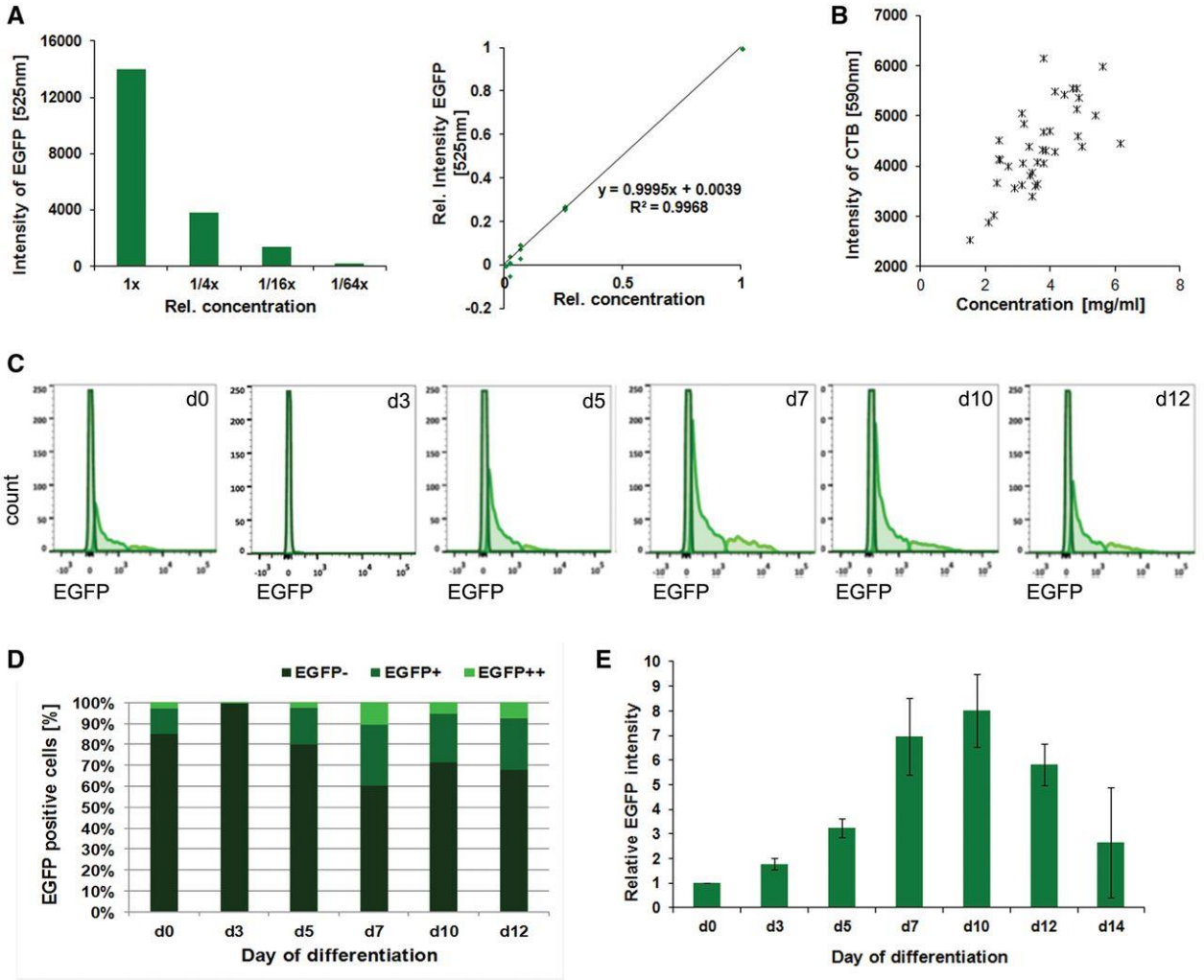


Figure 2: Quantification of EGFP expression during the differentiation of BRE-ESCs. (A) Direct measurements of day 7 BRE-ESC lysate serial dilutions quantified with a Tecan Infinite M2000 plate reader (excitation: 485 nm, emission: 535 nm, left). The graphical analysis of the fluorescence dependent on the relative protein amount allowed the extrapolation of a linear relationship with a high R^2-value. (B) Scatter plot of protein concentrations, calculated from absorption at 280 nm versus CTB measurements (excitation: 560 nm, emission: 590 nm) of cell lysates generated from replicates of the same experiment indicating significant correlation. (C) Quantification of the relative number of low and high EGFP expressing cells in the course of differentiation by FACS analysis. (D) Quantification of the relative number of low and high EGFP expressing cells in the course of differentiation calculated from results obtained in (C). (E) Quantification of EGFP expression from lysates generated from 25 EBs each at the indicated time points. The amount of EGFP expression was standardized against the protein amount determined by OD_{280} measurements and normalized against the EGFP expression of day 0.

As an alternative experimental approach, we analyzed EGFP expression via fluorescence activated cell sorting (FACS). Here, we could verify our previous results. In undifferentiated ESC cultures, already 15% EGFP-positive cells could be detected. However, no EGFP expressing cells were found at day 3 of differentiation, whereas EGFP expression was induced again at day 5 and reached its maximum at day 7, with over 40 percent of the cells being EGFP-positive (Fig. 2C, D). At later stages roughly 30 percent of the cells maintained a high level of EGFP expression. In addition, the FACS analysis revealed that the absolute amount of EGFP expression varied over a wide range among EGFP-positive cells indicating the formation of distinct cell populations. Applying EGFP measurements in cellular lysates, we obtained comparable results. Significant EGFP expression was induced between day 5 and day 7 of differentiation. Again, the highest activity could be found between day 7 and day 10, whereas in later stages of differentiation the EGFP expression dropped again (Fig. 2E). The discrepancy between FACS and lysate measurements of EGFP expression, especially seen on day 3, might be explained with the autofluorescence from dead cells which are commonly found during the differentiation process. In contrast, FACS analysis allowed to gate for living cells, thereby reducing potential, unspecific autofluorescence. In sum, the analysis of EGFP expression by Western blot, qPCR, FACS, and fluorescence measurements in cellular lysates strongly correlated and confirmed a differentiation-dependent expression of the EGFP transgene. Importantly, FACS analysis of BRE-ESCs allowed the quantification of the number of EGFP-positive cells as well as the identification of cell populations with distinct EGFP expression levels, whereas the general EGFP expression could easily be determined by fluorescence measurement in cellular lysates. The latter being particularly interesting, since it greatly facilitates the analysis and quantification of effects of chemicals on cellular differentiation and Bmp signaling activity.

Stage-dependent inhibition of EGFP expression and cardiomyocyte differentiation by a Bmp antagonist

To confirm the Bmp dependence of the EGFP expression, we analyzed the effect of the specific Bmp inhibitor LDN193189 on EGFP expression and cardiomyocyte differentiation. LDN193189 was recently shown to interfere with the activity of Bmp type I receptors with even higher specificity than the previously identified inhibitor Dorsomorphin (Vogt *et al*., 2011). Continuous exposure of differentiating BRE-ESCs to various LDN193189 concentrations revealed a significant reduction of EGFP expression and a dramatic reduction of cardiomyocyte differentiation already at 5nM (Fig. 3A), thereby confirming the specificity for EGFP expression as a Bmp target gene as well as the importance of Bmp signaling for the differentiation of mesodermal derivatives. Similar experiments with SB431542, a specific inhibitor for Tgfβ signaling via Smad2 and 3, resulted in impaired cardiac differentiation, but did not significantly reduce EGFP expression (Fig. S1). We further could verify inhibition of Id1 and Smad6, two direct Bmp target genes, by LDN193189

at the mRNA level (Fig. 3B). The mRNA of these two target genes was similarly down regulated in a comparable dose-dependent manner as EGFP. Additionally, we could detect repression of mesodermal (Gata4), endodermal (Afp) and the endothelial cell lineages (CD31/ Pecam1), whereas neural differentiation seemed to be stimulated as indicated by the enhanced expression of the neural marker gene Map2 (Fig. 3B).

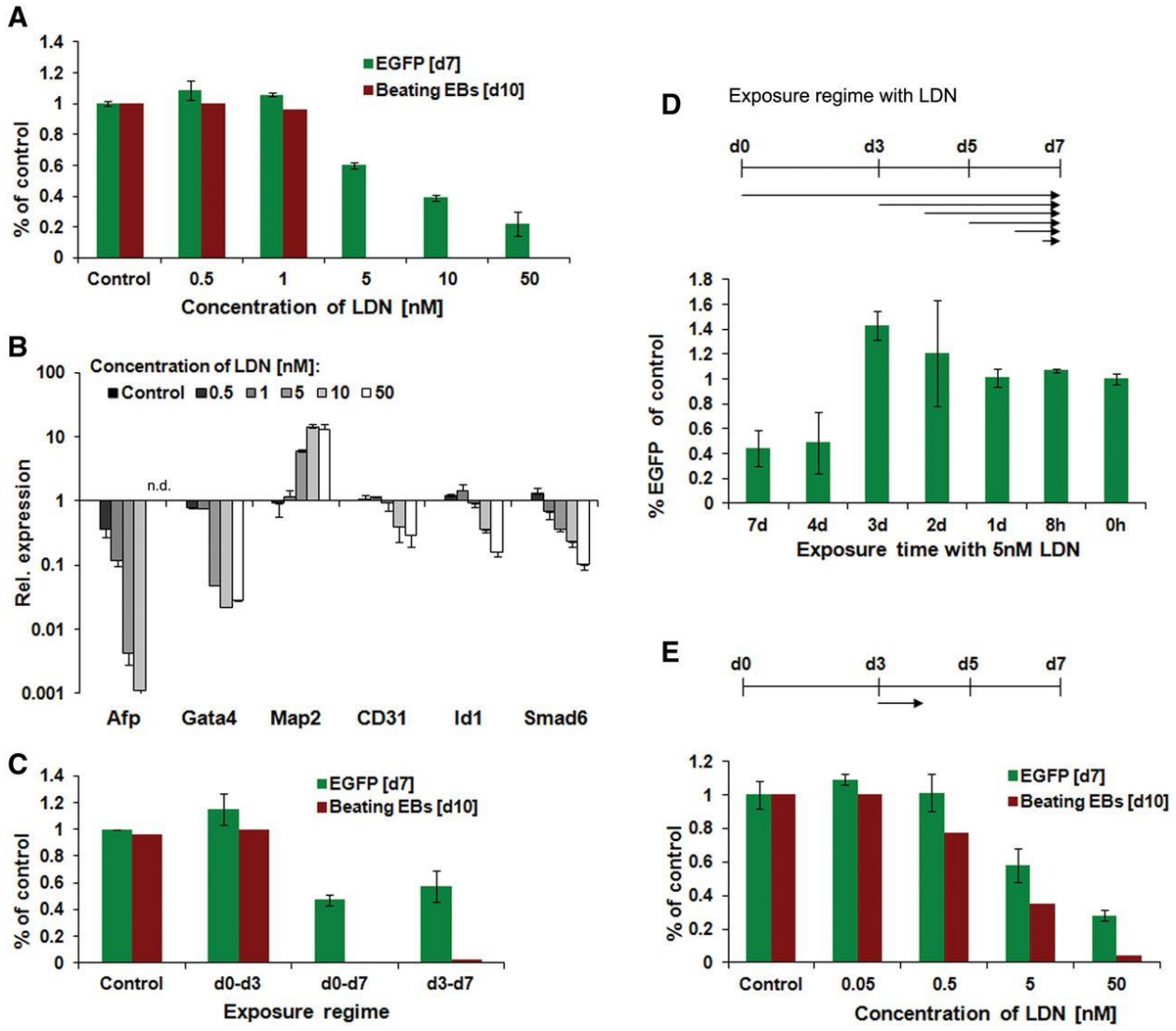
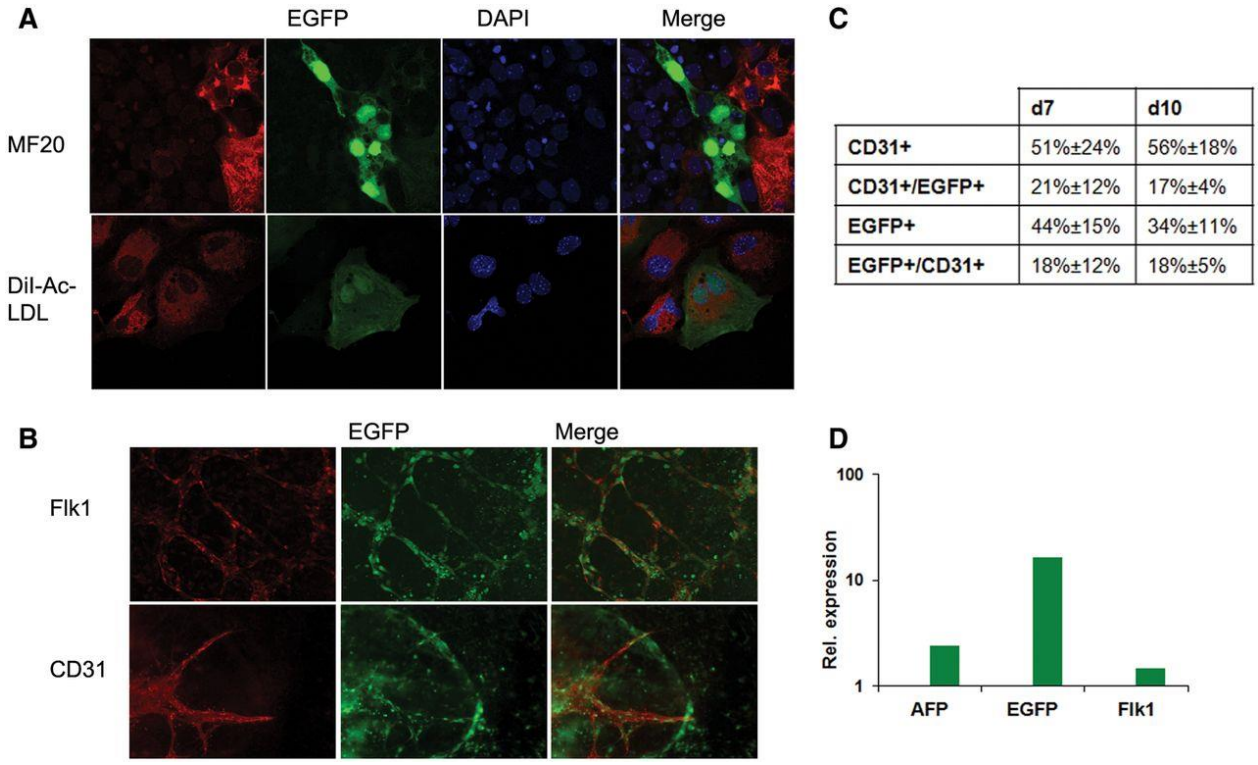


Figure 3: Dose-dependent inhibition of EGFP expression and cardiomyocyte differentiation by LDN193189. (A) Dose-dependent effect of LDN193189 on EGFP expression (green bars) and the differentiation of cardiomyocytes (red bars). Contracting areas were no longer detectable at 5nM LDN193189 but significant EGFP expression could still be found in cell lysates. (B) qPCR analysis of LDN193189 effects on the differentiation process at day 7 of differentiation. The expression of the indicated marker genes were standardized against Gapdh expression and normalized against the untreated control. (C) Determination of the inhibitory effect of LDN193189 on EGFP expression at day 7 (green bars) and cardiomyocyte differentiation at day 10 (red bars) after exposure from day 0-7, 0-3, and 3-7, respectively. (D) The effect on EGFP expression (green bars) and cardiac differentiation (red bars), with significant reduction of both endpoints with 5nM LDN193189.

To further confirm the time point of Bmp induction within the differentiation process, we applied the inhibitor LDN193189 at 5nM during different time periods (Fig. 3C). Interestingly, this inhibitor does not affect differentiation when applied only between day 0 and day 3: neither EGFP expression nor the formation of beating cardiomyocytes was reduced. In contrast, the differentiation is efficiently impaired when the inhibitor is applied continuously for 7 days. Moreover, exposure between day 3 and day 7 was sufficient to elicit the strong inhibitory effect of LDN193189 on EGFP expression and cardiomyocyte formation (Fig. 3C). To further narrow down the critical time frame, we exposed cells to 5nM LDN193189 from day 0, 3, 4, 5, 6 or 7 of differentiation onwards (Fig. 3D). LDN193189 did not affect EGFP expression when applied later then day 3 of differentiation (Fig. 3D). Although the expression of EGFP peaked at day 7 (Fig. 2D), we were unable to chemically interfere with the induction of EGFP expression after day 3. So, the presence of LDN193189 during the short time window between day 3 and day 4 of differentiation is required to inhibit EGFP expression in differentiating BRE-ESCs. To confirm this, a dose response experiment was performed restricting the exposure of differentiating BRE-ESCs against LDN193189 to the time window of day 3 through day 4 of differentiation (Fig. 3E). This short exposure time resulted in a reduction of EGFP expression that was comparable with the inhibition after continuous exposure from day 0 onward. Similarly, the differentiation into cardiomyocytes could be effectively inhibited by 5nM LDN193189 after transient exposure between day 3 and day 4, although the inhibitory effect of LDN193189 was somewhat less effective (Fig. 3A and 3E). Together, these results indicate that inhibition of EGFP expression is highly dependent on a specific time window that correlates with mesoderm induction. After the induction of mesoderm and concomitant EGFP expression, LDN193189 can no longer interfere with EGFP expression. The relatively long half-live of EGFP protein (Corish and Tyler-Smith, 1999) might contribute but can hardly explain this effect on its own. Importantly, EGFP expression and cardiomyocyte differentiation again correlated nicely. Further, our qPCR results suggest that EGFP-positive cells might contribute to the endodermal and endothelial cell lineages (Fig. 3B).

Characterization of EGFP-positive cells

To confirm the identity of the EGFP-positive cells as being distinct from the beating cardiomyocytes, we analyzed day 10 EBs using confocal microscopy after staining for MF20, that is, a marker for myosin heavy chain in cardiac and skeletal muscle. As expected the two cell types are in close proximity, but none of the EGFP-positive cells were positive for MF20 (Fig. 4A, upper panel).



	d7	d10
CD31+	51%±24%	56%±18%
CD31+/EGFP+	21%±12%	17%±4%
EGFP+	44%±15%	34%±11%
EGFP+/CD31+	18%±12%	18%±5%

Figure 4: EGFP-positive cells differentiate into endothelial and endodermal cells. (A) Fluorescence images of day 10 EB outgrowth stained with anti-MF20 antibodies (upper panel, red) and DiI-Ac-LDL (lower panel, red). Co-localization of EGFP fluorescence (green) with MF-20 could not be detected. Nuclei were stained with DAPI (blue). (B) Day 12 EBs stained with anti-Flk1 (upper panel, red) and anti-CD31 antibodies (lower panel, red) show partial co-localization with EGFP fluorescence. (C) Quantification of EGFP and CD31 single and double positive cells by FACS analyses at day 7 and day 10 of differentiation, each were calculated against total cell numbers. (D) qPCR analyses of EGFP-positive and EGFP-negative cells sorted by FACS. Samples were first standardized against Gapdh expression and normalized against the EGFP-negative cell population.

The net-like structures that were formed by the EGFP-positive cells at day 10 to day 12 of development indicated that these cells might develop into endothelium. Thus, we analyzed the expression of early endothelial markers, in particular vascular endothelial growth factor receptor 2 (Flk1/Vegfr2) and CD31, in immunohistochemical studies (Fig. 4B). Indeed, some of the EGFP-positive cells forming the net-like structures simultaneously expressed Flk1 and CD31. We then tested whether the endothelial cells are functional and display the typical increased metabolism of acetylated low density lipoproteins (Ac-LDL) that can be detected after addition of Ac-LDL conjugated to the carbocyanine dye DiI (DiI-Ac-LDL) to the medium (Voyta *et al.*, 1984). DiI-Ac-LDL was added for 4h and the cells analyzed by confocal microscopy. As can be seen in the lower panel of Fig. 4A, differentiating EBs developed cells that took up DiI-Ac-LDL and some of these cells were also positive for EGFP supporting the assumption that some of the EGFP-positive cells might indeed form functional endothelial cells.

Next, FACS was used to confirm that some of the EGFP-positive cells are committed to endothelial lineage. As shown in Fig. 4C, about half of the cells at day 7 and day 10 of differentiation expressed CD31. In addition, a significant proportion of the cells were EGFP-positive at day 7 (44 %) and day 10 (34 %). Most importantly, almost half of the CD31 positive cells were also found positive for EGFP at day 7 and vice versa. In addition, we used FACS to separate EGFP-positive from EGFP-negative cells and compared these two cell populations by qPCR analysis. Thereby we confirmed that purified EGFP-positive cells displayed increased levels of Flk1 as well as Afp mRNA compared with the EGFP-negative cells, although the increase in expression with respect to Flk1 was rather low (Fig. 4D). Thus, a significant number of the EGFP-positive differentiated toward endodermal and endothelial fate. Our results indicate that the EGFP reporter might be suitable for the analysis of vascularisation and angiogenesis *in vitro.*

Time-dependent effects of VPA and CHIR99021 on EGFP expression and cardiomyocyte differentiation

We compared the activity of valproic acid (VPA) and the specific Gsk3ß inhibitor CHIR99021 on the expression of EGFP and cardiomyocyte differentiation. We first tested the activity of 100 µg/ml VPA during different time periods, a concentration that has been described before to inhibit cardiomyocyte differentiation efficiently (Genschow *et al*., 2004). As expected, VPA efficiently inhibited EGFP expression and cardiomyocyte differentiation when applied for 7 days (Fig. 5A). However, the inhibition was similar if the cells were exposed to VPA for the first 3 days of differentiation only. In contrast, exposure to VPA after day 3 did not significantly affect EGFP expression or cardiomyocyte differentiation. Thus, VPA exposure during the early phase of differentiation is necessary and sufficient for VPA to exert its inhibitory effects, indicating that VPA interferes with differentiation processes before Bmp signaling is induced.

We compared the time-dependent activity of VPA with the specific chemical Gsk3ß inhibitor CHIR99021. Gsk3ß is a central component of the Wnt signal transduction pathway and inhibition of Gsk3ß is mimicking active Wnt signaling in many aspects. Here, we were able to demonstrate that increased Wnt signaling also interferes with the differentiation of cardiomyocytes as well as EGFP expression. Interestingly, similar to VPA, inhibition of Gsk3ß by CHIR99021 for 3 days sufficed to reduce EGFP expression, whereas treatment from day 3 of differentiation onward had no significant effect on the general EGFP expression level (Fig. 5B). However, already at day 7, we observed a change in the morphology of the EGFP-positive cells that clustered over the entire outgrowth (Fig. 5D). This suggested altered cell fate through increased Wnt signaling at later stages of differentiation that was not seen after VPA treatment (Fig. 5C). In addition, CHIR99021 treatment impaired cardiac differentiation at all time windows tested. Thus, in comparison with VPA treatment, the stimulation of Wnt signaling by inhibition of Gsk3ß activity had some similar but also certain distinct effects on BRE-ESC differentiation. These results imply that VPA and CHIR99021 exert

overlapping but also distinct activities, a result that further emphasizes the potential value of the BRE-ESCs in the analysis of the molecular mode of action of teratogenic substances. In addition, morphological evaluation of the EGFP-positive cells might provide an additional endpoint for the assessment of potentially toxicological effects. Our results also show that EGFP expression and cardiomyocyte differentiation do not necessarily correspond. Instead, EGFP expression has been established as an independent endpoint. Overall, application of BRE-ESCs enable for comparative analysis and characterization of the mode of action of chemical substances during the process of tissue differentiation.

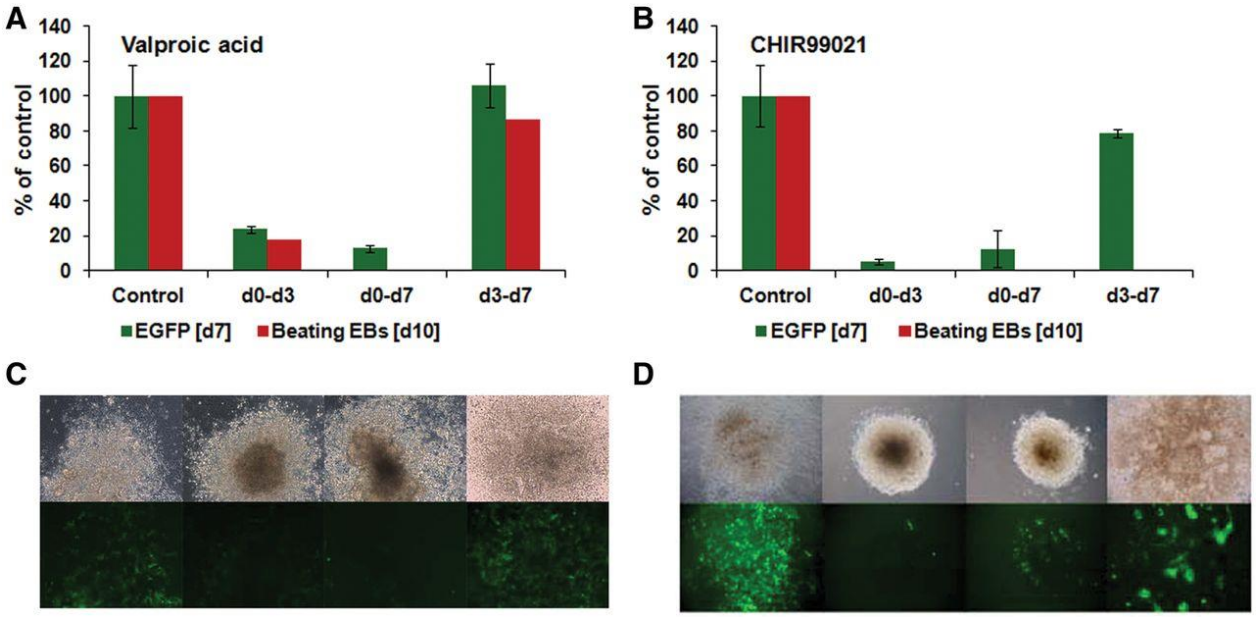


Figure 5: Time-dependent effects of VPA and CHIR99021. Effects of the exposure of differentiating BRE-ESCs with 100 µg/ml VPA (A) or 3 µM CHIR99021 (B) for 3 days, 7 days or during day 3 to day 7 of differentiation. At day 7, EGPF levels were quantified by fluorescence measurements in cellular lysates (green bars) and cardiomyocyte differentiation was assessed microscopically at day 10 (red bars). The microscopic appearance of the treated cells at day 7 is depicted in C and D for VPA (C) and CHIR99021 (D), respectively (brightfield in the upper panel, EGFP fluorescence of the same EB outgrowth in the lower panel).

Comparison of the dose response of known teratogenic substances on EGFP expression and cardiomyocyte differentiation

Finally, we wanted to compare the dose dependence of the effects of known teratogens on the expression of EGFP with the classical endpoint of cardiomyocyte formation. We selected retinoic acid (RA), 6-aminonicotinamid (ANA) and VPA because these substances have been well characterized in the classical EST and have been shown to act via distinct mechanisms. We performed dose response experiments in which we determined the differentiation into cardiomyocytes on day 10 and the expression of EGFP on

day 7 (Fig. 6A, B, C). For all three teratogens, both endpoints were significantly affected at comparable concentrations that correlated with previously described ID_{50} values from classical EST studies (ID_{50} for VPA: 50±8µg/ml, RA: 0.8±0.7ng/ml and ANA: 1.0±0.3µg/ml) (Genschow *et al.*, 2004). Thus for the tested substances, the impairment of differentiation can be evaluated with both methods, whereas the EGFP method provides the advantage that the measurements can be done 3 days earlier.

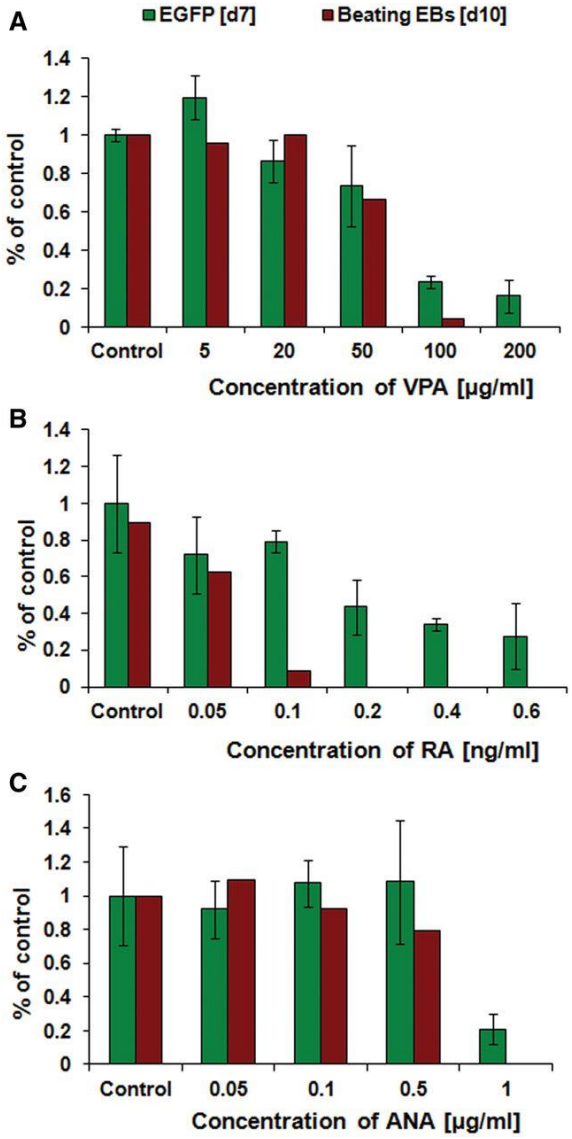


Figure 6: Dose-dependent effects of VPA, RA and ANA on EGFP expression and cardiomyocyte differentiation. Treatment of differentiating BRE-ESCs with the indicated concentrations of (A) valproic acid (VPA), (B) retinoic acid (RA) and (C) 6-aminonicotinamide (ANA) and subsequent effects on EGFP expression (green bars) and cardiomyocyte differentiation. EGFP expression was quantified at day 7 and cardiomyocyte differentiation analyzed at day 10 of differentiation (red bars).

Discussion

Here, we describe the characterization of an ESC line isolated from mice carrying a transgenic EGFP reporter gene under control of a Bmp response element as tool for the *in vitro* analysis of Bmp-dependent processes and the characterization of the teratogenic activities of chemical compounds. The transgene has been previously characterized *in vivo* as a reliable tool to follow Bmp signaling *in vivo* (Monteiro *et al*., 2008). Bmp signaling contributes to the formation of various organs and tissues in the course of vertebrate embryogenesis (Zhao, 2003) and, as expected, EGFP transgene expression broadens at later stages of development. We show that BRE-ESC reporter cells reproducibly differentiated into cardiomyocytes using the hanging drop method with kinetics comparable to the ESC line D3 described earlier (Seiler and Spielmann, 2011). Low levels of EGFP expression were already detectable in pluripotent BRE-ESCs before induction of differentiation correlating with the observation that Bmp target genes of the Id (inhibitor of differentiation) family are expressed in undifferentiated ESC cultures in the presence of fetal calf serum (Ying *et al*., 2003). Within 10 days, we observed robust cardiac differentiation as well as significant expression of EGFP, with almost half of all cells being EGFP-positive already after day 7 (Fig. 2 and 4). The differentiation-dependent expression of EGFP correlated well with the expression of known Bmp target genes and pSmad1 levels in the developing EBs coinciding with the time of mesoderm formation (Winnier *et al*., 1995), as well as with EGFP expression observed in the transgenic mice *in vivo* (Monteiro *et al*., 2008). Similar as in the reporter mice, the EGFP-positive cells formed different cell types during *in vitro* differentiation, including endodermal and endothelial cells.

After 10 days of differentiation, immunohistochemistry indicated the formation of EGFP-positive net-like structures expressing the endothelial markers CD31 and Flk1 (Fig. 4). These results were confirmed in FACS analyses that verified the presence of EGFP/CD31 double positive cells. In addition, a subset of the CD31-positive cells was EGFP-positive and the mRNA levels of endothelial marker genes were subtly increased in the EGFP-positive cell population. This is in line with the observation that the expression of EGFP *in vivo* is confined to a specific subset of endothelial cells during the specification of stalk and tip cells probably due to the inhibitory effect of Notch signaling (Moya *et al*., 2012). A discrepancy between nuclear pSmad1/5/8 and EGFP-positive cells was also observed *in vivo* that could reflect the relatively long half-life of EGFP that interferes with a direct correlation with the highly dynamic changes in Smad phosphorylation (Corish and Tyler-Smith, 1999).

Recently, an *in vitro* model based on gene expression analysis has been described to characterize the effects of chemical teratogens on endothelial differentiation (Festag *et al*., 2007), generating similar results as obtained in the classical EST. We tested our system by applying a set of well characterized chemical

teratogens in dose response experiments and showed that the effects of these compounds on EGFP expression at day 7 correlated with the effects on cardiac differentiation at day 10 of differentiation (Fig. 6). Similar results were obtained using endothelial marker genes (Festag *et al*. 2007). However, it remains unclear whether this assay can actually extent the applicability domain of the EST. To this end it has to be analyzed in detail in future studies applying tissue specific teratogenic substances. On the other hand, analysis of high-throughput screening data originating from ToxCast compounds strongly suggests that vascular development is a suitable endpoint for the assessment of developmental toxicity and the characterization of modes of actions for teratogenic compounds (Kleinstreuer et al., 2011). Taken together, the BRE-ESCs provide a suitable tool for the analysis of both cardiomyocyte and endothelial cell differentiation.

Experiments with the specific Bmp inhibitor LDN193189 and the TGFβ inhibitor SB431542 supported the specificity of the reporter construct but also revealed additional aspects of cardiac differentiation (Fig. 3 and S1). LDN193189 induced neural (ectodermal) cell fate at the expense of mesendodermal differentiation. These effects were expected, since Bmp activity is required in the cellular differentiation of mesendodermal tissues and subsequent mesodermal differentiation (Van Vliet *et al*., 2012). Conversely, inhibition of Bmp signaling is essential for the determination of neural cell fate *in vivo* (Bier *et al*., 2011). The strongest effects of LDN193189 treatment were observed on endodermal marker genes and qPCR analysis following cell sorting indicated that EGFP-positive cells were partially endodermal (Fig. 4). As discussed by Rana and coworkers (Rana *et al*., 2013), differentiation of the cardiac mesoderm *in vivo* depends on the underlining endodermal cell layer that expresses Bmp protein thereby inducing the expression of cardiac specific transcription factors such as Nkx2.5 and Gata4. *In vitro*, the endodermal gene Sox17 acting downstream of the early endodermal marker Afp was found to be required for the differentiation of primitive mesoderm into cardiac mesoderm (Liu *et al*., 2007). In our culture system, cardiomyocytes are EGFP-negative but the EGFP-positive cells were frequently found in the vicinity of beating cardiomyocytes (Fig. 4). Thus, it will be interesting to determine whether these cells are equivalent to the endodermal cells that control cardiac development *in vivo*. The induction of mesodermal fate in BRE-ESCs took place between day 3 and 4 of differentiation, and this was also found to be the most effective time window for LDN193189 to inhibit EGFP expression and the formation to both endoderm and mesoderm.

Based on our results, microscopic evaluation of EGFP-positive cells offers useful additional information to reveal the mode of action of certain chemicals (Fig. 5). For example, the Wnt agonist CHIR99021, an inhibitor of phosphorylation and subsequent degradation of β-catenin through Gsk3β, displayed a time-

dependent effect on EGFP expression. When cells were treated for the first 3 days or the entire time period of 7 days, the inhibitor markedly reduced EGFP expression and cardiac differentiation. Interestingly, when applied from day 3 onward, CHIR99021 alters cell fate noticeable in a changed morphology of EGFP-positive cells, which then cluster all over the EB outgrowth, indicating that CHIR99021 exerts distinct effects depending on the differentiation status. It also points to the independence of the EGFP endpoint, as cardiac differentiation can be impaired without loss of EGFP expression, an effect that was also seen with the specific TGFβ inhibitor SB431542 (Fig. S1).

These analyses of small chemical antagonists of BMP and Wnt signaling suggest that the BRE-ESC might also serve as valuable tool for the identification and characterization of chemical modulators of Bmp or Wnt activity in the frame of biomedical research. Cell lines as well as phenotypic screens in zebrafish have already been successfully applied for screening substances that specifically activate or inhibit these signaling pathways (e.g. Vrijens et al., 2012; Rennekamp and Peterson, 2014). The BRE-ESCs provide a distinct differentiation-dependent cellular context, are compatible with high-content and high-throughput screening approaches and rely on the endogenous, differentiation-dependent regulation of the BMP pathway. Future studies have to show if they can have a significant impact on these kinds of studies.

Similar to CHIR99021, VPA also has an effective time window between day 0 and day 3, pointing to a related mode of action of both compounds. Indeed, VPA has been proposed to inhibit Gsk3ß, mimicking active Wnt signaling (Chen *et al*., 1999), but also to act as histone deacetylase antagonist (Wiltse, 2005). Bmp and Wnt signaling are both essential for mesoderm induction during early embryonic (Loebel *et al*., 2003; Winnier *et al*., 1995) and subsequent cardiac development (Van Vliet *et al*., 2012). Interestingly, Bmp and Wnt signaling pathways can directly interact since Gsk3ß directly phosphorylates and inhibits Bmp-specific Smads (Fuentealba *et al*., 2007). In contrast to CHIR99021, however, VPA did not affect EGFP expression or cardiac differentiation at later stages of differentiation pointing to a distinct mode of action of VPA. The analysis of additional teratogens, in particular retinoic acid (RA) and 6-aminonicotinamide (ANA), demonstrated that EGFP measurement allows the characterization of teratogens with very different molecular mode of actions (Fig. 6). RA is known to act as ligand for RA (RAR) and retinoid X receptors (RXR) which both can heterodimerize and bind to regulatory elements in RA-responsive genes. Exogenous RA can interfere with the endogenous RA signaling leading to teratogenic effects (Horton and Maden, 1995; Mark *et al*., 2006). In contrast, ANA is thought to interfere with the pentose phosphate pathway by inhibiting glucose-6-phosphate dehydrogenase (Tyson *et al*., 2000).

Finally, our results also implicate BRE-ESCs as a valuable tool for high throughput analysis of potential teratogenic compounds in combination with high content imaging. Such an approach might allow EGFP

measurement in combination with the evaluation of the morphology and the cardiac differentiation of individual EBs at the same time. There are multiple ways to analyze the effects of chemical teratogens on signaling cascades. For instance, transfecting reporter constructs directly into ESC and evaluating its activity after exposure with teratogenic compounds, as has been described for the ReproGlow assay (Uibel *et al*., 2010). However, it seems important to keep in mind that the activity of artificial reporter gene constructs might be affected by its genomic integration site and not necessarily reflect corresponding *in vivo* activities (Wilson *et al*., 1990). This problem might be partially solved with the establishment of cell lines derived from transgenic animals for which *in vivo* information relating to the onset and tissue specific expression of the transgene is available. Reporter mouse lines have been already established for various evolutionarily conserved signaling pathways (e.g. Wnt, Notch) known to be involved in a plethora of developmental processes and in tissue homeostasis (Maretto *et al*., 2003; Nowotschin *et al*., 2013).

In summary, we demonstrated that BRE-ESCs represent a versatile tool to assess Bmp activity in ESC differentiation and the analysis of teratogenic effects of compounds *in vitro* using an established protocol for cardiomyocyte differentiation. Since Bmp signals play a pivotal role in the formation of various organs these cells might also be used to address other differentiation processes, including neurogenesis or osteogenesis, if specific differentiation protocols can be applied.

Funding information

This work was supported by the Interuniversity Attraction Poles-Phase VII [IUAP/PAI P7/14] to SMCdSL. Financial support via intramural funding of the BfR was provided to JK and MO (SFP No. 1322-422).

Acknowledgment

We thank C. Mummery for providing the transgenic mice and the critical reading of the manuscript and Riana Vogt (MPI-IE) for the generation of the BRE-ESC line.

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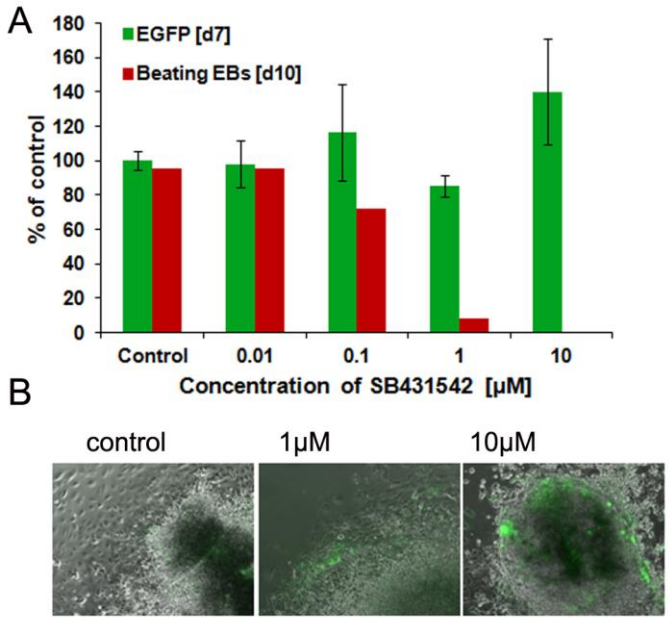
Supplementary



Supplementary Figure 1: Effect of SB431542: Effects of the exposure of differentiating BRE-ESCs with indicated concentrations of SB431542 during differentiation. (A) At day 7, EGFP levels were quantified by fluorescence measurements in cellular lysates (green bars) and cardiomyocyte differentiation was assessed microscopically at day 10 (red bars). The microscopic appearance of the treated cells at day 7 is depicted (B).

3.2 Identification and characterization of teratogenic chemicals using embryonic stem cells isolated from a Wnt/β-Catenin-reporter transgenic mouse line

Similarly to the first study addressing Bmp signaling, we investigated whether the Wnt/β-Catenin signaling pathway can be used to predict teratogenic activities. From the BAT-Gal mouse line, the laboratory of Prof. R. Kemler (MPI-IB) generated ESCs which were kindly provided for this study. The BAT-Gal-ESCs were somewhat slower to differentiate into functional cardiomyocytes, but otherwise expressed all markers in the expected order. Nevertheless, I could use he BAT-Gal-ESCs to analyze the impact of teratogens on the canonical Wnt signaling pathway and establish this cell line as an additional test system for the identification and characterization of teratogenic activities.

In order to detect the reporter gene β-Galactosidase (LacZ) we applied different staining reagents: (1) X-Gal, as described in section 1.3.3.2, for *in situ* staining of the ESCs or differentiated EBs; (2) ortho-Nitrophenyl-β-D-galactoside (ONPG) for quantitative measurement of the yellow cleavage product ortho-nitrophenol production in cell lysates; and (3) C12-FDG (5-dodecanoylaminofluorescein di-β-D-galactopyranoside), which is cleaved into a green fluorescent product making it possible to detect reporter protein positive cells *in vitro* in flow cytometry and microscopy. All three detection methods resulted in comparable results and revealed an induction of endogenous, canonical Wnt signaling at day 5 of differentiation correlating with the expression of Brachyury and, thus, mesoderm induction. Enrichment of reporter gene positive cells by fluorescence activated cell sorting (FACS) revealed the mesodermal character of LacZ positive cells. Interestingly, the enriched cells also expressed Snail, an essential marker for epithelial to mesenchymal transition.

With an inhibitor of Tankyrase (XAV939) and, thus, inhibiting the intracellular transmission of the Wnt signal, and an inhibitor of Gsk3β (CHIR99021) that artificially induces nuclear translocation of β-Catenin mimicking active Wnt signaling, I could verify the specificity of the reporter gene and define an essential time window for the regulation of reporter gene expression. Using the same set of model substances, I could also verify that induction as well as repression of the reporter gene activity, detected either on day 5 or day 7 of differentiation, can be used as a predictive indicator for teratogenic activities.

The author's contribution:

Design of experimental approach, conduction of experiments, evaluation, and interpretation of experimental data

Preparation of manuscript and figures

Editor's Highlight: Identification and characterization of teratogenic chemicals using embryonic stem cells isolated from a Wnt/β-Catenin-reporter transgenic mouse line

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Toxicological Sciences 2016 Aug;152(2):382-94

doi: 10.1093/toxsci/kfw094

Identification and characterization of teratogenic chemicals using embryonic stem cells isolated from a Wnt/β-Catenin-reporter transgenic mouse line

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Abstract

Embryonic stem cells (ESCs) are commonly used for the analysis of gene function in embryonic development and provide valuable models for human diseases. In recent years, ESCs have also become an attractive tool for toxicological testing, in particular for the identification of teratogenic compounds. We have recently described a Bmp-reporter ESC line as a new tool to identify teratogenic compounds and to characterize the molecular mechanisms mediating embryonic toxicity. Here we describe the use of a Wnt/β-Catenin-reporter ESC line isolated from a previously described mouse line that carries the LacZ reporter gene under the control of a β-Catenin responsive promoter. The reporter ESC line stably differentiates into cardiomyocytes within 12 days. The reporter was endogenously induced between day 3 to 5 of differentiation reminiscent of its expression *in vivo*, in which strong LacZ activity is detected around gastrulation. Subsequently its expression becomes restricted to mesodermal cells and cells undergoing an epithelial to mesenchymal transition. The Wnt/β-Catenin-dependent expression of the reporter protein allowed quantification of dose- and time-dependent effects of teratogenic chemicals. In particular, valproic acid reduced reporter activity on day 7 while retinoic acid induced reporter activity on day 5 at concentrations comparable to the ones inhibiting the formation of functional cardiomyocytes, the classical read-out of the embryonic stem cell test (EST). In addition, we were also able to show distinct effects of teratogenic chemicals on the Wnt/β-Catenin-reporter compared to the previously described Bmp-reporter ESCs. Thus different reporter cell lines provide complementary tools for the identification and analysis of potentially teratogenic compounds.

Key words: Wnt, differentiation, stem cells, LacZ, reporter gene, embryo

Introduction

The Wnt/β-Catenin signal transduction pathway regulates a plethora of different biological processes in embryonic development and adult tissue homeostasis and mutations in the Wnt/β-Catenin signaling pathway have been implicated in a large variety of human diseases including cancer (Clevers and Nuesse, 2012, MacDonald et al. 2009, Ring et al. 2014, Yang et al. 2016). The Wnt protein family consists of about 19 family members that can bind to a heteromeric receptor complex consisting of a seven transmembrane protein of the frizzled family and an Lrp5/6 protein. Activation of this pathway leads to the inhibition of the β-catenin destruction complex, β-catenin accumulation, and translocation of β-catenin protein into the nucleus where it can modulate gene transcription in concert with Lef/Tcf transcription factors (MacDonald et al., 2009). The Wnt/β-Catenin signaling pathway has been highly conserved in evolution. Components of this pathway have been identified throughout the animal kingdom and its function, in particular in respect to the determination of the anterior-posterior or oral-aboral body axis, seems to be conserved as well (Petersen and Reddien, 2009). In addition, the Wnt/β-Catenin signal transduction pathway is a key player in stem cell and cancer stem cell biology and thus an important potential target for cancer therapy (Clevers et al., 2014; Kahn and Kim, 2014).

Given the importance of the Wnt/β-Catenin pathway for various physiological and pathological conditions it is not surprising that it has been intensively studied and various tools have been developed to analyze Wnt/β-Catenin activity *in vitro* as well as *in vivo*. In particular, a number of reporter mouse lines have been established to monitor Wnt/β-Catenin signaling activity in the developing embryo or in adult tissues (Currier et al., 2010 and references therein). Reporter mice have become increasingly important, not only for basic research but also for drug testing and toxicological risk assessment. This is because they offer simple tools to detect changes in signaling activity upon exposure to a drug or chemical in a time- and dose-dependent manner under physiological conditions (Maggi and Ciana, 2005; Boverhof et al., 2011). Cell lines that have been stably transfected with a reporter gene construct offer an even simpler, more cost-effective and high-throughput compatible alternative to the *in vivo* models, although they do not provide a physiological tissue environment. In addition, the expression of a reporter gene might be affected by genomic sequences surrounding its integration site (Wilson et al., 1990) and, thus, might not always correspond to *in vivo* signaling activities. Nevertheless, Wnt/β-Catenin -reporter cell lines have already been successfully established and used for drug screening as well as for toxicological testing (Barker and Clevers, 2006; Uibel et al., 2010). To combine the advantages of the *in vivo* and *in vitro* approaches, we have recently described the establishment of a test system based on an ESC line that had been derived from transgenic mice carrying a Bmp-reporter gene construct (Monteiro et al., 2008). We

could verify that this approach is suitable to identify and characterize teratogenic substances applying a standardized protocol for cardiomyocyte differentiation (Kugler et al., 2015).

Here we describe an ESC line, BAT-Gal-ESCs (β-catenin activated transcription of β-galactosidase), derived from transgenic mice that carry the LacZ reporter gene encoding β-galactosidase (β-Gal) under the control of a Wnt/β-Catenin regulated promoter (Maretto et al., 2003). In the transgenic mice, expression of the transgene was first detected in the primitive streak and subsequently in somites, the cardiac anlagen, neural crest, and the mid and hind brain regions nicely correlating with known regions of high Wnt/β-Catenin activity. Here we show that the transgene is endogenously induced during the differentiation of the BAT-Gal-ESCs into cardiomyocytes, being mainly expressed in mesodermal derived cells and cells undergoing epithelial to mesenchymal transition (EMT) later on. At early stages of differentiation, the transgene is expressed during the induction of mesoderm, a process that is known to be highly dependent on active Wnt/β-Catenin signaling. Interference with the differentiation process by exposure to specific chemical inhibitors or known teratogenic substances alters the expression of β-Gal in a predictive and quantitative manner. Our results demonstrate that the BAT-Gal-ESC line is also suitable for the identification of teratogenic substances. However, the effects observed in the BAT-Gal-ESCs differ from the results obtained with the previously described Bmp-reporter ESCs and imply that a combinatorial use of these two cell lines can provide even more mechanistic information about potentially teratogenic substances and will be particularly useful to complement already existing test systems.

Material and Methods

ESC culture

BAT-Gal-ESCs were isolated from blastocysts of the Wnt/β-Catenin-reporter mice (Maretto et al., 2003) as previously described (Doetschman et al., 1985) Experiments were performed in agreement with the German law on the use of laboratory animals as well as biosafety (S1) and institutional guidelines of the Max Planck Society. The use of animals was approved by “Regierungspräsidium Freiburg (Freiburg regional council)” and the animal welfare office of the Max Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany (KE-2iTO-6). For the cultivation of the cells 2i conditions were applied as described elsewhere (Kugler et al., 2015).

Differentiation protocol

The BAT-Gal-ESCs were differentiated using the hanging drop method (Seiler and Spielmann, 2011) with minor changes. In brief, 20 µl droplets of cultivating media without leukemia inhibitory factor (LIF)

containing 1000 cells were incubated for three days. At day 3, after embroid body (EB) formation, the EBs were rinsed and either single EBs were cultivated in 24-well plates for evaluation of functional differentiation into cardiomyocytes for additional 9 days or groups of 20 EBs were cultivated in 6-well cell culture plates for RT-PCR or analysis of β-Gal activity until day 5 or day 7 of differentiation, respectively.

In situ staining

The X-Gal staining was performed using β-Galactosidase Reporter Gene Staining Kit (Sigma-Aldrich) according to manufacturer's protocol. In brief, the EBs were rinsed with PBS and fixed for 3 min at room temperature. After two washing steps with PBS the EBs were incubated with staining solution for 45 to 60 min at 37°C and the staining intensity was monitored by light microscopy. As a complementary method for the detection of β-Gal activity, EBs were incubated with media containing 30 µM C_{12}-FDG (5-dodecanoylaminofluorescein di-β-D-galactopyranoside; Fisher Scientific) for 60 min at 37°C (Debacq-Chainiaux et al., 2009). This staining provides the advantage of detection of β-Gal activity in living cells and allowed the isolation of β-Gal positive cells by FACS analysis.

qPCR

Total RNA extraction was performed using the Trizol reagent (Life Technologies) as described in the manufacturer's protocol. To ensure complete removal of genomic DNA, a DNase step was performed for 30 min using 5 µl DNase Buffer and 1 µl DNase (NEB England) for 45 µl of total RNA at 37°C. The reaction was stopped on ice and the RNA subsequently purified by phenol/chloroform extraction and ethanol precipitation. Two micrograms of total RNA were used for cDNA synthesis using the HighCapacity cDNA Reverse Transcription Kit (Life Technologies) according to the manual. After diluting the cDNA 1:10 in water, 1 µl was used for each qPCR reaction using a 7500 Fast Real-Time PCR system (Life Technologies). PCR primer sequences can be found in Supplementary Table 1. Gene expression of target genes (TG) was standardized against three house-keeping genes (*Gapdh*, *Ppia*, and *Pgk1; HG*) using the geometric mean of their C_T-values (Pfaffl et al., 2004) and normalized against the appropriate control:

$$relative\ Expression = \frac{2^{C_T(TG_{Sample})}}{\frac{2^{\sqrt[3]{C_T(HG1_{Sample})*C_T(HG2_{Sample})*C_T(HG3_{Sample})}}}{2^{C_T(TG_{Control})}}}{2^{\sqrt[3]{C_T(HG1_{Control})*C_T(HG2_{Control})*C_T(HG3_{Control})}}}$$

If not stated differently, standard deviation (SD) was calculated from biological triplicates with differences > 3 x SD considered statistically significant.

FACS

Prior to fluorescence activated cell sorting (FACS) of C_{12}-FDG stained EBs, the EBs were treated with TrypLE Express (Life Technologies) and EDTA (Pan, Germany) for 30 min. To create a single-cell solution, the cell suspension was vigorously pipetted with FACS buffer (PBS with 5% FCS and 1% EDTA) and filtered through cell strainer caps (Fisher Scientific). To discriminate dead from living cells, cell suspension was stained using the Live/Dead fixable near infrared staining kit (1:1000, Life Technologies) for 30 min at room temperature. FACS was performed using a FACS Aria III (BD Biosciences). Collected cell fractions were either stored in Trizol at -20°C for subsequent total RNA extraction or transferred on slides and fixed in 4% of paraformaldehyde for 10 min for immunofluorescence.

Immunofluorescence

After permeabilization with 0.5% Triton-X-100, fixed cells were blocked with block buffer (5% BSA in PBST) for 30 min at room temperature. The anti-Snail (abcam, ab180714; 1:500 in block buffer) primary antibody was incubated for 60 min at room temperature and appropriate secondary antibody coupled to Cy3 (Jackson ImmunoResearch Laborytory; 1:200 in block buffer) for 30 min. Intensity measurements were done using ImageJ and statistical analysis was done with a pairwise Wilcoxon test.

Chemicals

All chemicals used were purchased from Sigma-Aldrich, except of acrylamide (from Biorad) and CHIR99021 and XAV939 (axon Medchem). All substances were dissolved in PBS (valproic acid, acrylamide, dephenhydramine, lithiumchlorid, LDN193189) or DMSO (6-aminonicotinamide, retinoic acid, CHIR99021, XAV939). The solvent concentration was the same for all dilutions of a test substance and the solvent control.

β-Gal assay

The β-Gal assay was performed using the Mammalian beta-Galactosidase Assay Kit (Fisher Scientific). In short, cells or differentiating EBs were trypsinized for 15 min and pooled. After centrifugation, the pellet was lysed in 50 µl M-PER Mammalian Protein Extraction Reagent and incubated over night at 4°C. The protein content of the centrifuged lysate was assessed through the absorption at 280 nm using a Nano-Drop. Thirtyfive microliter volumes of the lysate were transferred into a 96-well plate and mixed with 35 µl Pierce beta-Galactosidase Assay Reagent containing *ortho*-nitrophenyl-β-galactoside. Subsequently the formation of reaction product *o*-nitrophenol was monitored through measuring the optical density at 405 nm (OD_{405nm}) every 5 min for 2.5 h. The slope of product formation against time was calculated,

standardized against protein content of the sample, and normalized against the appropriate control. Where not stated differently, standard deviation (SD) was calculated from biological triplicates. Significance testing was performed using a pairwise Wilcoxon test and calculated significances are indicated. Biological relevance was assumed when fold change was above 2 or below 0.5, respectively.

To ensure linear correlation between β-Gal activity and protein content, we performed a serial dilution series of samples with high β-Gal activity, after treatment with CHIR99021 during differentiation, and correlated β-Gal activity against the relative protein amount of the samples.

Dose-response curves

The dose-response curves for cardiac differentiation and protein content were performed using a three parametric fit of the "drc" package in R on the combined data of three independent runs (Ritz and Streibig, 2005; R Core Team, 2015). From the dose-response curves the concentration at which the differentiation into cardiomyocytes is impaired to 50%, the ID_{50}, and the concentration at which the protein content falls below 50% of the control could be calculated.

Results

Characterization of the differentiation of BAT-Gal-ESCs into cardiomyocytes

The BAT-Gal-ESCs could efficiently be differentiated into cardiomyocytes using a modified protocol of the embryonic stem cell test (EST; Seiler and Spielmann, 2011), in which EBs are generated by the hanging-drop method and plated on cell culture dishes on day 3 of differentiation. The differentiation process was subsequently analyzed applying qPCR as well as *in situ* staining, and detection of spontaneously beating cardiomyocytes by light microscopy at day 12 of differentiation.

As shown in Fig. 1A, the qPCR analysis confirmed a differentiation process comparable to the one observed with the previously described BRE-ESCs or D3 ESCs (Kugler et al., 2015; Seiler and Spielmann, 2011). The pluripotency marker Oct4 was downregulated, whereas marker genes for mesodermal (Brachyury) and endodermal (Afp) as well as cardiogenic precursor cells (Alcam and Gata4) were effectively upregulated. A significant stimulation of the reporter gene LacZ was only transiently detectable at day 5, corresponding nicely with the expression pattern of the direct Wnt target gene Brachyury and recapitulating the transient character of strong Brachyury expression during mesoderm induction *in vivo* as well as *in vitro* (Kispert and Hermann, 1994; Lako et al., 2001). The apparent high starting level for Brachyury and LacZ mRNA on day 0 might well be due to the cultivation of the BAT-Gal-ESCs in 2i media containing CHIR99021 (Ying et al., 2008), an inhibitor of Gsk3β that mimics active Wnt signaling and promotes pluripotency, but also

stimulates the transcription of direct Wnt target genes like Brachyury or the LacZ reporter. The microscopic evaluation of the differentiation process revealed that beating cardiomyoyctes can be detected in over 80% of the analyzed cultures at day 12 of differentiation, which is slightly later compared to D3 or BRE-ESCs. However, an efficiency of >80% effective cardiomyocyte differentiation over at least six consecutive experiments allowed us to use the BAT-Gal-ESCs as a reliable test system, in which teratogenic effects could be analyzed.

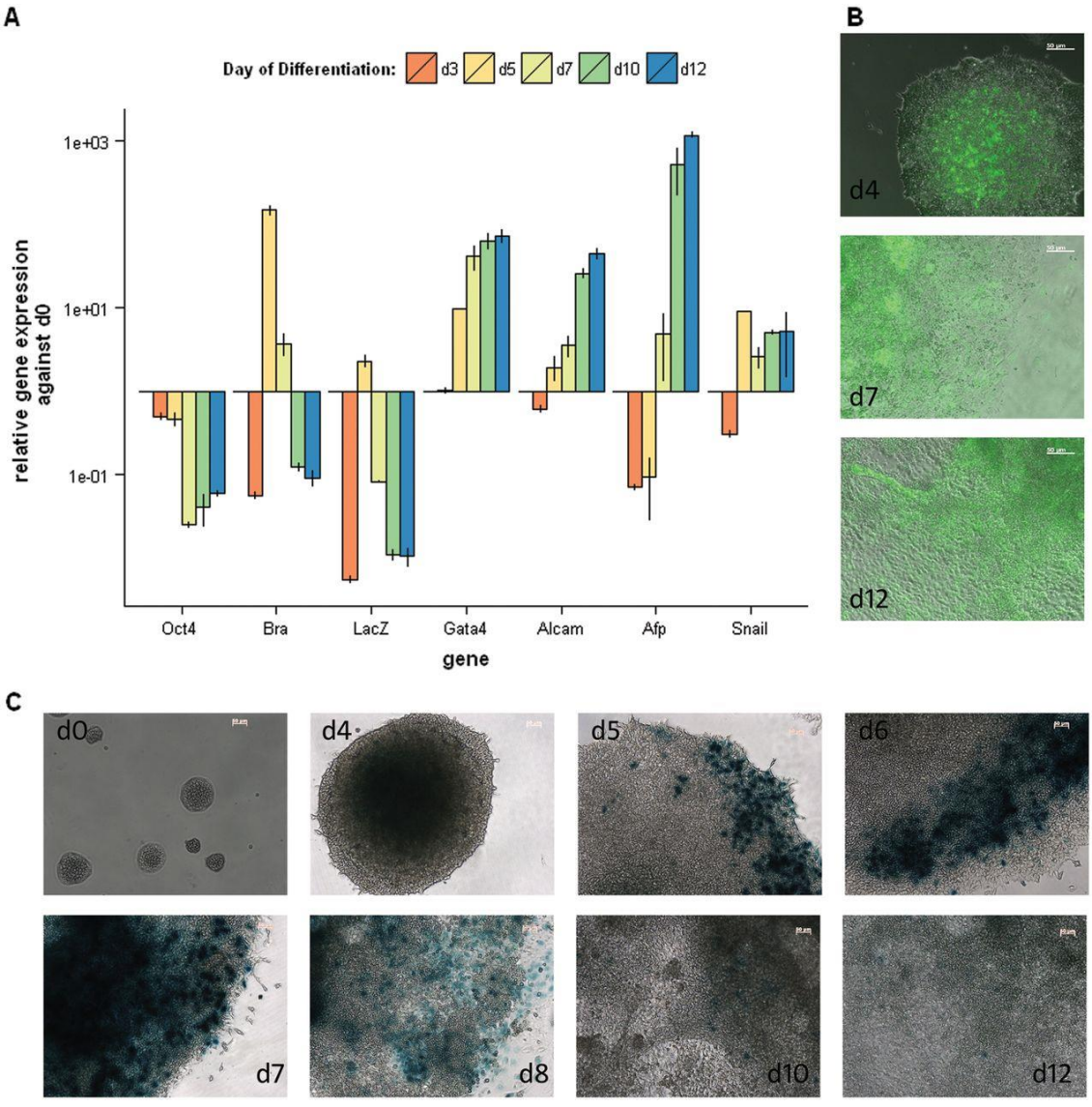

Figure 1: Characterization of BAT-Gal-ESCs. (A) qPCR analysis of specific marker genes during the course of differentiation. All samples are normalized against Gapdh, Pgk1, and Ppia, and standardized against d0. Afp is a marker for endodermal cells, Alcam and Gata4 for cardiac precursor cells, Brachyury (Bra) for mesodermal cells, LacZ is the reporter gene, Oct4 a marker for pluripotency, and Snail a marker for neural crest cells. (B) Staining of β-Gal activity with C12-FDG, detectable as green fluorescence, as overlay with light microscopic pictures of the outgrowths of EBs at the indicated time-points. (C) Light microscopic pictures of LacZ-staining with X-Gal (blue) of differentiating cells at indicated time-points.

We monitored the β-Gal expression through X-Gal staining (Fig. 1C), and were able to detect β-Gal-positive cells as early as day 5 of differentiation. The staining intensifies until day 7, indicating an increased expression that might also reflect β-Gal protein accumulation due to its rather long half-life (Gonda et al., 1989). The β-Gal-positive cells show a broad distribution over the entire EB with some cells migrating out of the EB center. In addition, we used C_{12}-FDG to monitor β-Gal-positive cells. This substrate is cleaved by β-Gal into a green fluorescent dye and can be used in living cells (Debacq-Chainiaux et al., 2009). This kind of staining is apparently more sensitive, since it allowed the detection of β-Gal-positive cells on day 4 of differentiation already (Fig. 1B). Although living cells can be monitored, the dye is also quite stable and accumulates in the cells, such that the staining cannot resolve time-dependent changes of β-Gal expression. However, both staining procedures gave comparable results, nicely correlating with the qPCR data, and thus verified the induction of reporter gene expression during mesoderm induction.

Quantification of the reporter gene

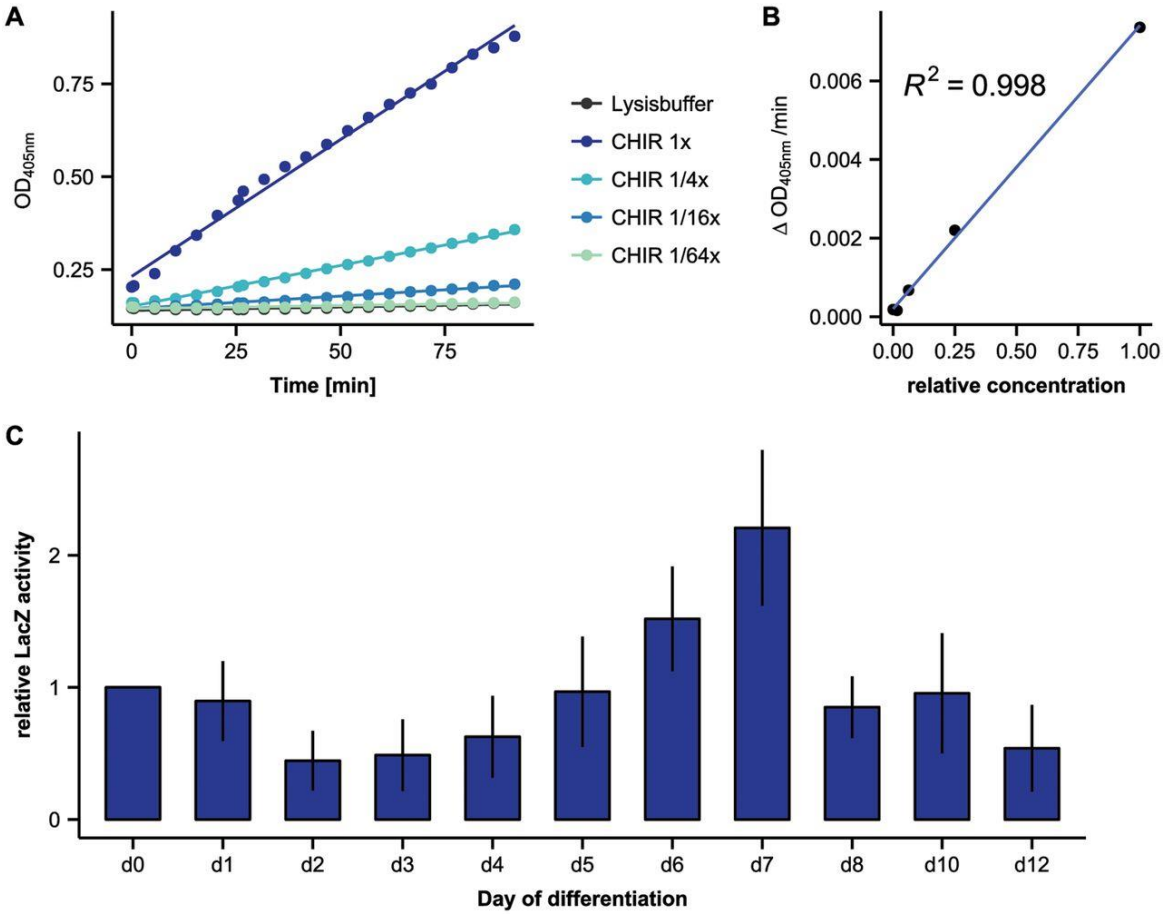

Figure 2: Quantification of β-Gal activity. (A) Determination of β-Gal activity in lysates by measuring the increase of the optical density at 405nm (OD_{405nm}) through the generation of the yellow product *o*-nitrophenol over time of cell lysates derived from EBs, differentiated for 5 days and treated with 3 µM CHIR99021 (CHIR). The slope (ΔOD_{405nm}/min) indicates the activity of serial

dilutions. (B) Correlation of the measured slope against the relative protein concentration. (C) Relative β-Gal activity in lysates over the differentiation process. All samples are standardized against the protein concentration and normalized against d0 (n=5).

To measure the β-Gal activity quantitatively, we prepared cell lysates of differentiated EBs treated with CHIR99021, for which we expected a high β-Gal activity, and monitored the time-resolved changes of the optical density at 405 nm (OD_{405nm}). The slope (ΔOD_{405nm}/min) of the linear part of the resulting curve was used as a direct measure for β-Gal protein levels (Fig. 2A and B). Indeed, measuring the activity in serial dilutions, we could show that the slope directly correlates with the relative protein amount (R^2=0.998). The general protein content of the samples was assessed by measurement of the absorption at 280 nm and these values were then used to standardize the samples for varying amounts of protein in the cellular lysates as described before for the BRE-ESCs (Kugler et al., 2015). When monitoring the β-Gal activity over the differentiation process using this approach, we were able to detect a slight decline until day 3, followed by an endogenous induction starting at day 4 with a peak of activity on day 7, and a subsequent reduction (Fig. 2C). These data nicely recapitulated the findings from our qPCR analysis and *in situ* staining. Thus, using three different experimental approaches we were able to characterize and to quantify the endogenous Wnt signaling activity in the BAT-Gal-ESCs during differentiation that correlated with the well-known function of Wnt signals in mesoderm induction.

Specificity of the reporter gene construct in vitro

The endogenous induction during *in vitro* differentiation correlates with the *in vivo* findings of the mouse model (Maretto et al., 2003). Additionally, we tested whether the reporter gene construct can also be used to detect external influences on the Wnt signaling activity. We applied two specific chemical inhibitors: (1) CHIR99021, inhibiting Gsk3β, thereby inducing Wnt signaling and β-catenin accumulation within the cells, and (2) XAV939, which inhibits tankyrase activity, preventing the dissociating of the destruction complex and the accumulation of β-catenin in the cytosol, ultimately inhibiting active Wnt signaling (Huang et al., 2009). Both substances were applied over the complete differentiation time and β-Gal activity was quantitatively assessed in the samples (Fig. 3A). The effect of these compounds on reporter gene activity during the differentiation process was found to be stage-dependent. While CHIR99021 (3 µM) effectively induced reporter gene expression during the first five days of differentiation prior to the endogenous induction of the reporter gene, this induction did not increase the level of endogenous induction and to some extent even reduced the endogenous levels later on. On the other side, XAV939 (1 µM) did not have any effect on β-Gal activity during the first five days, but inhibited the endogenous induction very efficiently in the later course of differentiation. Both substances inhibited the differentiation into

functional cardiomyocytes at day 12 effectively at the concentrations used. Although the treatment with XAV939 and CHIR99021 resulted in impaired differentiation into cardiomyocytes, the morphological alterations of the outgrowth of the EBs indicated different effects through the reduction or induction of endogenous β-Gal activity.

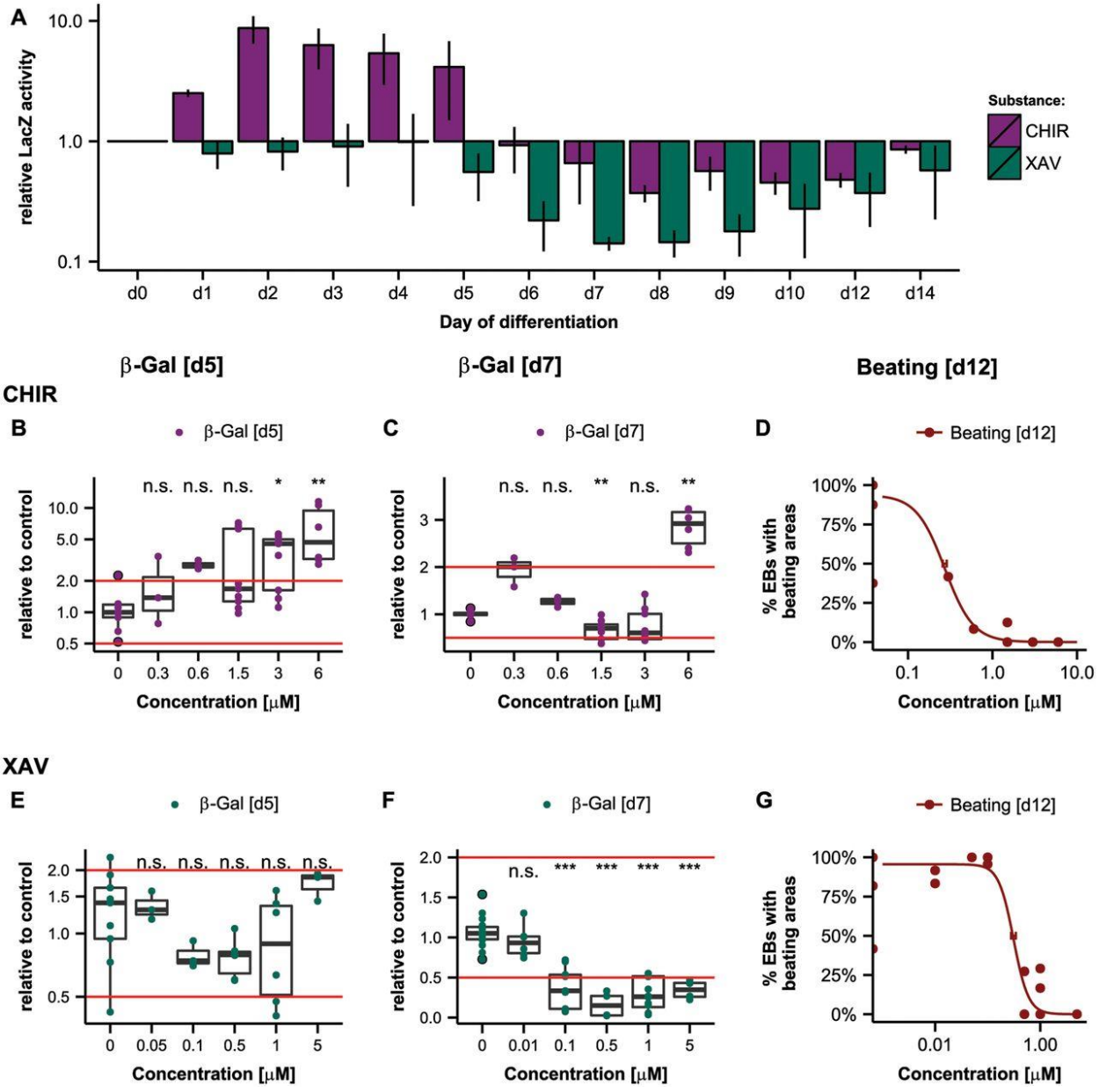

Figure 3: Specificity of reporter gene construct in vitro. (A) Effect of Wnt-modulating compounds on β-Gal activity over the differentiation process. Differentiating cells were incubated with CHIR99021 and XAV939 (3 µM and 1 µM) during the entire time of differentiation. β-Gal activity was determined in lysates at indicated time-points. All samples are normalized against the protein concentration and standardized against the appropriate solvent control (n=5). Dose-response curves for CHIR99021 (B, C, D) and XAV939 (E, F, G) on the β-Gal activity on day 5 (B, E), on day 7 (C, F), and cardiomyocyte differentiation on day 12 (D, G). All β-Gal activity samples are normalized against protein concentration and standardized against the appropriate solvent

control. Significance was calculated with a pairwise wilcoxon test of treated samples against control (n.s. = not significant, *: p < 0.05, **: p < 0.01, ***: p < 0.001). Biological relevance was defined as a fold change in β-Gal activity higher 2 or below 0.5 compared to control, respectively, to account for biological variation in our test system. These limits are indicated as red lines.

Next, we analyzed the effect of increasing concentrations of both substances on the β-Gal activity on day 5 and day 7 of differentiation and on cardiac differentiation on day 12. Here, we used a fold change of 2 or 0.5 in β-Gal activity as cut-off values for biologically relevant alteration of the differentiation process, thus taking into account an activity window for the experimental variation of β-Gal activity (red lines in Fig. 3). Additionally, we analyzed whether the results obtained are significantly different from the respective control. CHIR99021 increases the β-Gal activity dose-dependently on day 5 of differentiation. Comparing these effects with the inhibition of cardiac differentiation, the latter seems to be more sensitive being effected at lower concentrations (0.3 µM as ID_{50} for cardiac differentiation in comparison to 0.6 µM for a 2-fold increase in β-Gal activity). Taking into account that day 5 might be the turning point, where the endogenous induction of β-Gal activity cannot be raised any further by CHIR99021, the increase in β-Gal activity might be more pronounced at day 4 of differentiation. Nevertheless, we could observe a nice correlation between inhibition of cardiac differentiation and increased β-Gal activity also on day 5. However, analyzing β-Gal activity on day 7, no clear dose-dependent effects of CHIR99021 could be observed as the β-Gal activity can be reduced or induced, depending on the used concentrations (Fig. 3B-D). In contrast, XAV939 did not significantly alter β-Gal activity on day 5, but an efficient and dose-dependent decrease in β-Gal activity was detected on day 7 correlating with the inhibition of cardiac differentiation. Here, the reduction of β-Gal activity was even more sensitive than inhibition of cardiac differentiation with an ID_{50} of approximately 0.3 µM compared to 0.1 µM for a fold change under 0.5 compared to control β-Gal activity (Fig. 3E-G). From these results we concluded that inhibition as well as induction of reporter gene activity can be correlated with impaired differentiation. In addition, these data implied that an analysis of substance effects on day 5 and day 7 of differentiation is required in order to detect significant and dose-dependent stimulatory or inhibitory effects on the endogenous Wnt/β-Catenin activity level. Overall, for specific agonists as well as antagonists of Wnt/β-Catenin activity, the β-Gal activity, analyzed either on day 5 or day 7, correlated with the effects observed on the differentiation of cardiomyocytes that is already commonly used as a predictive endpoint for the identification of teratogenic substances. Thus the quantification of β-Gal activity might indeed provide a new, quantitative and predictive tool for the identification and characterization of toxic substances.

Identity of reporter gene positive cells

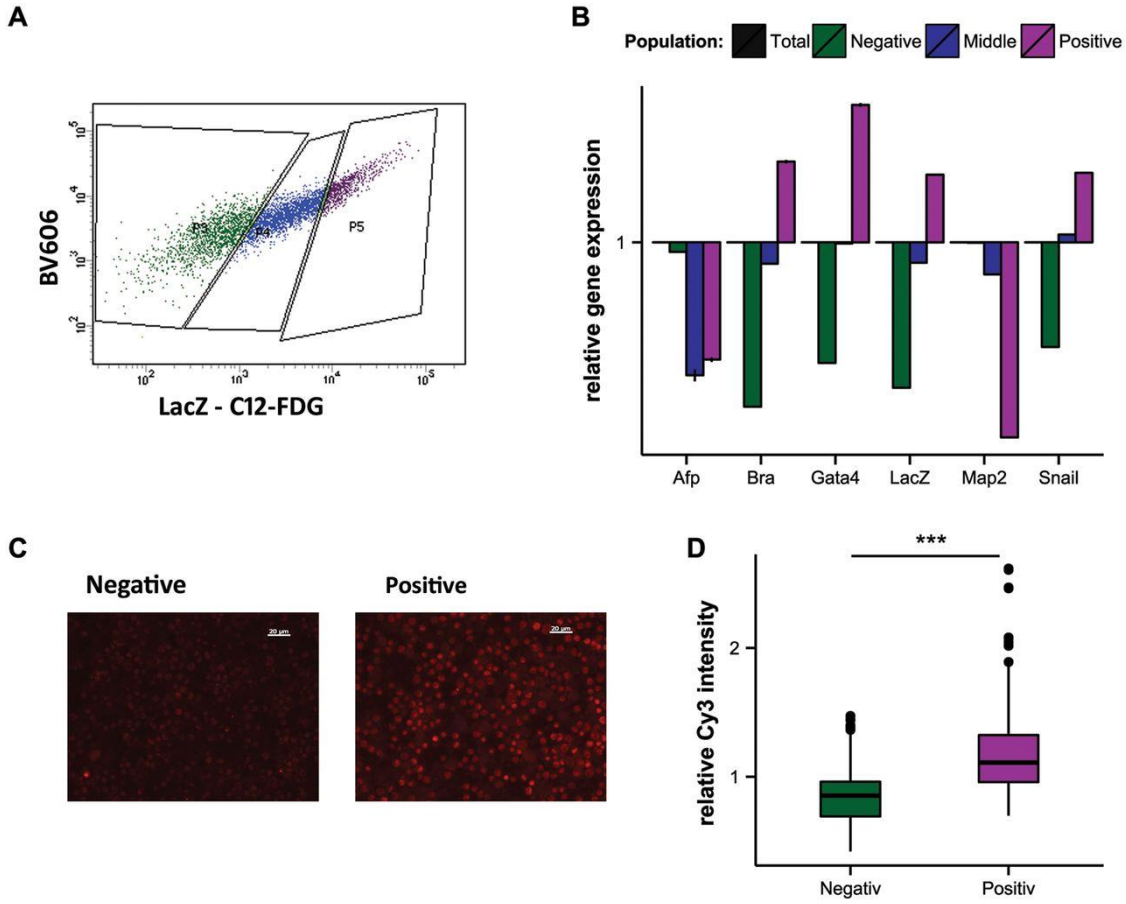

Figure 4: Identification of LacZ-positive cells. (A) Dot-plot of differentiating cells at d7 stained with C12-FDG (x-axis) against auto-fluorescence (y-axis). Indicated are the sub-populations of LacZ-negative cells (green), LacZ-positive (purple) and a middle-population (blue), that were separated using FACS. (B) qPCR of specific marker genes of sub-populations after sorting. Samples were normalized against Gapdh, Pgk1, and Ppia and standardized against the total cell population. Depicted are the results for Afp (endodermal cells), Brachyury (Bra, mesodermal cells), Gata4 (cardiac precursor cells), LacZ (the reporter gene), Map2 (neuronal cells), and Snail (marker for cells undergoing EMT). Depicted are the results of a representative experiment. (C) Representative pictures of Snail-staining (red, Cy3) of sub-populations (LacZ-negative and LacZ-positive) after cell sorting. (D) Statistical analysis of relative Cy3 intensity of Snail staining after cell sorting (*: $p < 0.001$).**

To characterize the system in more detail, we wanted to identify the reporter gene positive cells within the differentiating EBs. Cells were stained with C_{12}-FDG and subpopulations separated by FACS on day 7 of differentiation excluding dead cells and cell aggregates (not shown). Three subpopulations were isolated: negative cells, positive cells, and a middle population with weak fluorescent signal (Fig. 4A). These cells were subsequently analyzed by qPCR for ectodermal (Map2), mesodermal (Brachyury/Bra, Gata4), endodermal (α-fetoprotein, Afp) marker genes as well as for the gene Snail that is early expressed in the primitive streak and specific for cells undergoing epithelial to mesenchymal transition (EMT, Fig. 4B). The

very distinct β-Gal transcript levels in the three subpopulations verified the overall success of the sorting process. Brachyury and Gata4 as well as Snail transcripts were found to be significantly enriched whereas Afp and Map2 were hardly detectable in the fluorescence-positive cell population. These data suggest that the expression of the reporter gene is mainly restricted to cells of the mesodermal lineage and cells undergoing EMT as important aspect of differentiation. To confirm these results on the protein level, we performed immunostaining for Snail after cell sorting (Fig. 4C) and could also observe a significant difference between fluorescent and non-fluorescent cell populations. Given that the formation of mesoderm as well as the induction of EMT is highly dependent on Wnt signaling (Lim and Thiery, 2012), the BAT-Gal-ESCs used in our assay seem to recapitulate these well-known embryonic processes *in vitro.*

Predictivity of BAT-Gal-ESCs

To evaluate the capability of the BAT-Gal-ESCs to predict teratogenic effects, we applied teratogenic (retinoic acid (RA), 6-aminonicotinamide (6-AN), valproic acid (VPA), Lithiumchlorid (LiCl), Fig. 5 and 6, respectively) and non-teratogenic (acrylamide (AA), diphenhydramine (DPH), Fig. 6) substances (Brown 2002). LiCl was tested as an additional positive substance, as its Wnt/β-Catenin inducing effects are well described. In addition, we tested LDN193189, a specific Bmp-signaling inhibitor to test for cross-talk between the BMP and Wnt signaling pathways (Kugler et al., 2015). For all these substances we generated dose-response curves to estimate effective dose values for the β-Gal activity on day 5 and day 7 and evaluated whether the chemical-dependent changes in β-Gal activity were statistically significant and biologically relevant, taking into account the intrinsic variations of our test system. In addition, ID_{50} values for the inhibition of cardiomyocyte differentiation evaluated on day 12 were calculated and the protein content of the samples determined, as a measure for cytotoxic effects (Fig. S1). The final results of three biological replicates are summarized in Table 1.

Even for this relatively small test panel, we observed substance specific alterations of β-Gal activity. VPA and LDN193189 reduce the β-Gal activity on day 7 under 0.5-fold of the control at lower concentrations than the calculated ID_{50} for cardiomyocyte differentiation. These effects were also below concentrations exhibiting a measurable effect on the general protein content (see Fig. S1). Both substances stimulated β-Gal activity on day 5, but only at concentrations with clearly reduced protein content probably reflecting non-specific effects (marked with grey boxes in Fig. 5 and 6, respectively). RA and LiCl induced the β-Gal activity on day 5 markedly at concentrations at which no differentiation inhibition or reduction of protein content could be found. Interestingly, RA also induces the β-Gal activity at day 7, although less pronounced, whereas LiCl reduces the β-Gal activity on day 7 at concentrations comparable to the ID_{50} for cardiomyocyte differentiation. Thus, LiCl can have agonistic as well as antagonistic effects depending on

the time point of analysis. 6-AN, AA, and DPH only have effects on β-Gal activity at at concentrations clearly reducing the protein content in the samples, also indicating that these effects were likely unspecific (compare grey boxes in Fig. 5 and 6, respectively).

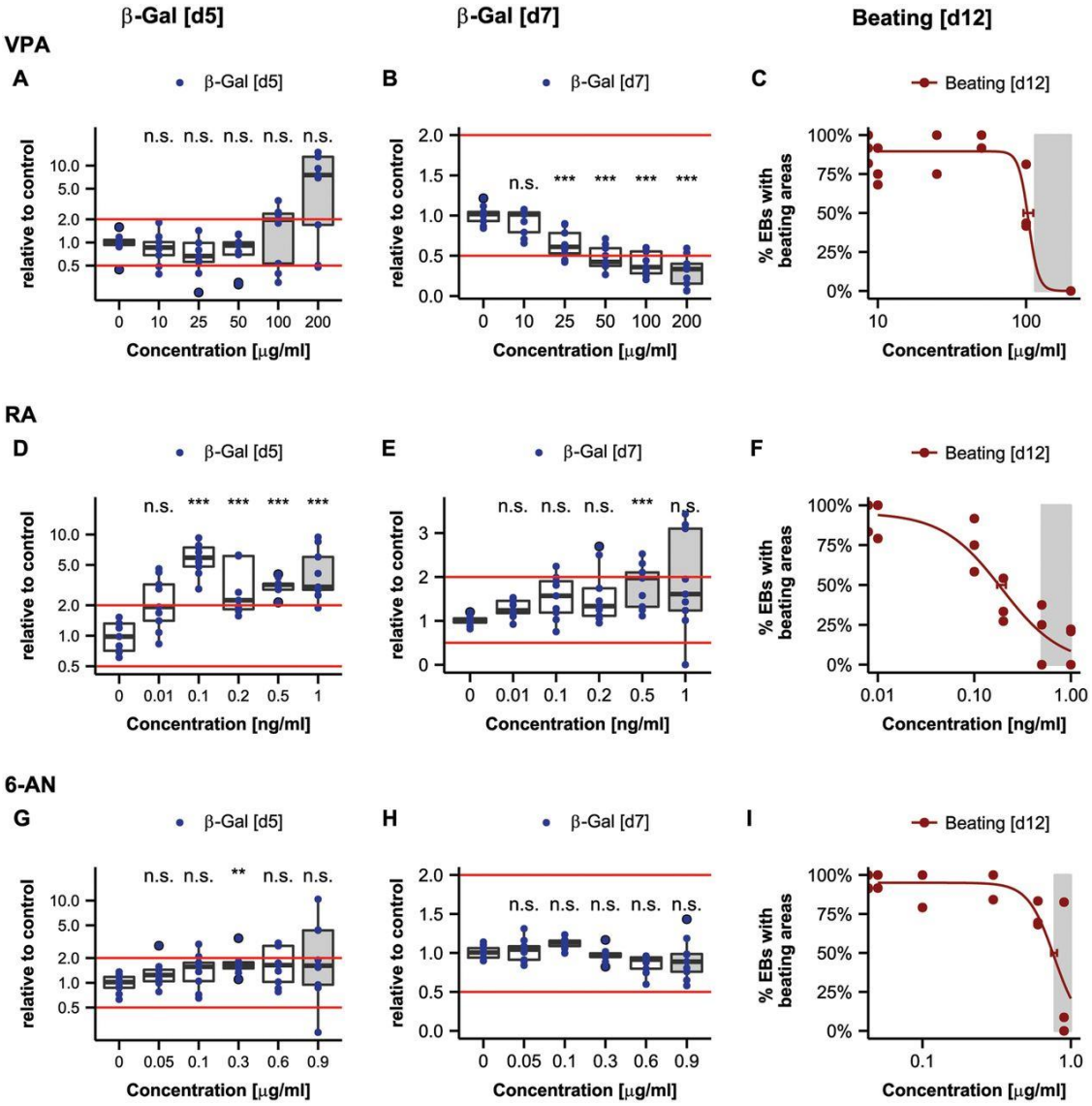

Figure 5: Dose-response curves for VPA, RA and 6-AN. Dose-response curves for valproic acid (VPA; A, B, C), retinoic acid (RA; D, E, F), and 6-aminonicotinamide (6-AN; G, H, I) on the β-Gal activity on day 5 (A, D, G), on day 7 (B, E, H), and cardiomyocyte differentiation on day 12 (C, F, I). All β-Gal activity samples are normalized against protein concentration and standardized against the appropriate solvent control. Significance was calculated with a pairwise wilcoxon test of treated samples against control (n.s. = not significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$). Biological relevance was defined as a fold change in β-Gal activity higher 2 or below 0.5 compared to control, respectively, to account for biological variation in our test system. These limits are indicated as red lines.

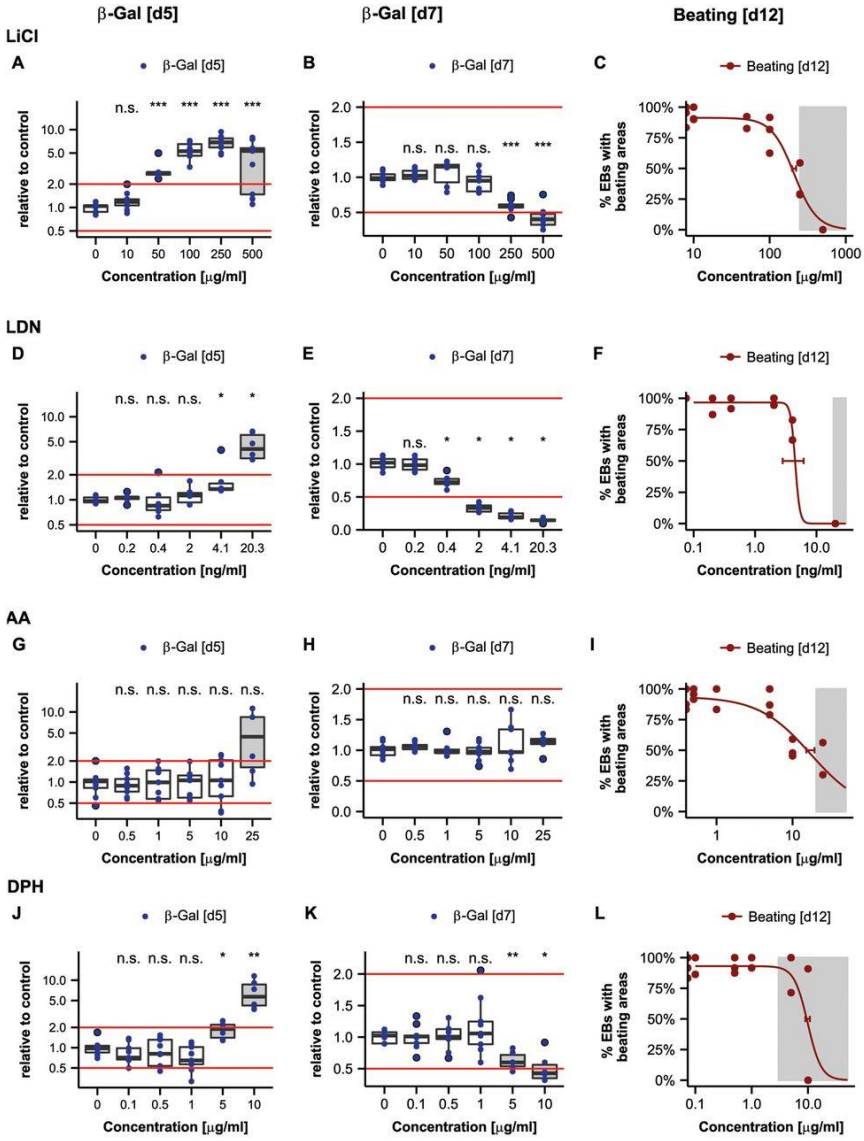

Figure 6: Dose-response curves for LiCl, LDN 193189, acrylamide and DPH. Dose-response curves for lithium chloride (LiCl; A, B, C), LDN193189 (D, E, F), acrylaminde (AA; G, H, I), and diphenhydramine (DPH; J, K, L) on the β-Gal activity on day 5 (A, D, G, J), on day 7 (B, E, H, K) and cardiomyocyte differentiation on day 12 (C, F, I, L). All β-Gal activity samples are normalized against protein concentration and standardized against the appropriate solvent control. Significance was calculated with a pairwise wilcoxtest of treated samples against control (n.s. = not significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$). Biological relevance was defined as a fold change in β-Gal activity higher 2 or below 0.5 compared to control, respectively, to account for biological variation in our test system. These limits are indicated as red lines.

Table 1: Effective doses for tested substances. Listed are effective doses for 2-fold induction of β-Gal activity on day 5 and for 0.5-fold induction of β-Gal activity on day 7, the calculated ID_{50} of cardiac differentiation on day 12, the IC_{50} of protein content in lysates on day 5 and day 7. Further, the effective doses for a 2-fold induction of the reporter gene activity in the ReProGlo assay (A Uibel et al., 2010) have been added.

Substance	β-Gal activity		ID_{50}	Protein content <50%		ReProGlo
	d5>2	d7<0.5	BAT-Gal-ESCs	d5	d7	BMC_2^A
VPA [µg/ml]	100	50	104 ± 8	99 ± 12	129 ± 13	30 ± 10
RA [ng/ml]	0.1	/	0.19 ± 0.02	0.7 ± 0.4	0.3 ± 0.1	1.5 ± 1.9
6-AN [µg/ml]	/	/	0.77 ± 0.04	0.87 ± 0.05	0.83 ± 0.06	/
LiCl [µg/ml]	50	500	220 ±10	200 ± 50	300 ± 20	243 ± 34
LDN [ng/ml]	20.3	2	4.5 ± 1.6	14 ± 11	21 ± 5	/
DPH [µg/ml]	10	10	6.5 ± 1.1	3 ± 1	3 ± 1	/
AA [µg/ml]	25	/	15.6 ± 1.5	19 ± 4	22 ± 2	/

Discussion

In this study, we analyzed the Wnt/β-Catenin signaling pathway as predictive tool to study the effects of teratogenic substances *in vitro*. BAT-Gal-ESCs expressing the LacZ gene under the control of seven Tcf/Lef binding sites were generated from transgenic mice in which tissues and cells associated with high Wnt/β-Catenin signaling activity during developmental processes could be detected (Maretto et al., 2003). The multi-step process of cardiac differentiation of ESCs was successfully used to predict teratogenic activities of selected compounds (Genschow et al., 2004). Here we combined both approaches in a BAT-Gal-ESC test system and established a quantitative Wnt/β-Catenin signaling activity test as a new read-out to identify teratogenic substances.

During the differentiation, all three germ layers are generated and spontaneously beating cardiomyocytes could be observed at day 12 in a reliable manner. The expression of LacZ correlated with the expression of the direct Wnt/β-Catenin target gene Brachyury, a marker for mesoderm induction. On the protein

level, β-Gal activity could also be detected via *in situ* X-Gal or C_{12}-FDG staining, indicating a broad distribution of reporter protein positive cells in differentiating EBs. Although mRNA of marker genes for cardiac mesoderm (Gata4 and Alcam), ectoderm (Snail), as well as endoderm (Afp) were upregulated during differentiation, the expression of β-Gal was mainly restricted to mesodermal cells and cells undergoing EMT, thereby reproducing *in vivo* data in which the reporter gene was detected in the primitive streak at day 7 p.c. (Maretto et al., 2003). This implies that we monitor at least two different cell populations when analyzing the effect of teratogens on day 5 and day 7. The importance of EMT during *in vivo* development of the heart is thoroughly documented, but the precise mechanisms during *in vitro* differentiation remain elusive (Kim et al. 2014, Van Vliet et al. 2012, von Gise und Pu 2012).

During cardiac differentiation Wnt/β-Catenin signaling has a biphasic role: Being essential for the induction of mesoderm, it has to be downregulated for the specification of cardiac mesoderm (Ueno et al., 2007). In our test system the reporter protein is induced upon mesoderm induction from day 3 onward the activity peaks at day 7. In the later course of differentiation it stays on a low level with only few cells being positive for the reporter protein – also nicely correlating with its mRNA levels. Thus, endogenous Wnt/β-Catenin signaling is only of transient character in agreement with the transient character of mesoderm induction. Additionally, Wnt/β-Catenin signaling has a narrow activity window in which it performs its role. Decreasing or increasing Wnt/β-Catenin signaling *in vivo* either through knock out of β-catenin or expression of its stabilized form, respectively, both lead to early embryonic lethality (reviewed in Grigoryan et al., 2008). When Wnt/β-Catenin signaling is upregulated the epidermal cells of the ectodermal cell layer disintegrate because of their transdifferentiation into mesodermal cells (Kemler et al., 2004) and upon downregulation the three germ layers fail to form (Haegel et al., 1995). Interestingly, *in vitro*, both up- and downregulation of Wnt/β-Catenin signaling inhibit the differentiation, resulting in increased expression of pluripotency markers such as Nanog and Oct4 (Atlasi et al., 2013), or in keeping the cells in an epiblast stem cell status (Sumi et al., 2013), thereby hampering cardiac differentiation. While an artificial upregulation can be monitored during the first 5 days of differentiation, the downregulation is detectable from day 7 onward. All these results underline that well defined levels and timing of Wnt/β-Catenin signaling activity are necessary in the cells for proper embryonic development and differentiation. The BAT-Gal-ESC test system allows screening for an artificial induction of Wnt/β-Catenin signaling on day 5 and a downregulation on day 7 and both endpoints indicate an impaired cardiac differentiation and a teratogenic activity.

The substances tested to evaluate the predictivity of our test system comprised four teratogens (VPA, RA, 6-AN, LiCl), two negative substances (DPH and AA), and a Bmp inhibitor (LDN193189) (Brown, 2006; Vogt

et al., 2011). RA is described as strong teratogen, which is attributed to the fact that it is an endogenous signaling molecule during development. The best described mode of action is the signaling through RA and retinoid X receptors (RAR and RXR) altering the transcription of genes possessing RA regulatory elements (Mark et al., 2006). Recently, RA was also implicated in driving differentiation of ESCs through the activation of the FGF signaling pathway (Stavridis et al., 2010). Kennedy et al. could also show that premature and prolonged treatment with RA favors skeletal muscle progenitors at the expense of cardiac differentiation by inducing the expression of Wnt molecules (Kennedy et al., 2009). Our results recapitulate such an activity for RA, as the induction of β-Gal activity on day 5 is closely correlated with impaired cardiac differentiation (Fig. 5). In BRE-ESCs, the teratogenic effects of RA could also be detected, as it reduced Bmp-reporter activity during differentiation dose-dependently (Kugler et al., 2015). This is a nice example of an inhibitory or stimulatory effect of a teratogen depending on the particular reporter gene constructs that might complement each other in detecting and characterizing teratogenic activities.

VPA and LiCl are weak teratogens and implicated in altering Wnt/β-Catenin signaling via distinct mechanisms (Wiltse, 2005; Brown, 2002). LiCl is implicated in cardiac malformations (Weinstein and Goldfield, 1975); it inhibits Gsk3β thereby inducing Wnt/β-Catenin signaling (Klein and Melton, 1996). Here, it raises the reporter protein activity on day 5 markedly as expected, while reducing it on day 7 at concentrations also inhibiting cardiac differentiation (Fig. 6), thereby indicating a direct correlation of active Wnt/β-Catenin signaling with cardiac differentiation *in vitro*. VPA is described as histone deacetylase (HDAC) inhibitor, which raises the overall protein level of β-catenin within cells, but induces the reporter protein activity at relatively high concentrations only (Fig. 5). In contrast the reduction of β-Gal activity at day 7 was seen at concentrations not yet being inhibitory to cardiac differentiation. Thus, the inhibitory activity of VPA seems more relevant to explain its teratogenic activity. In contrast, in BRE-ESCs, we only detected an inhibitory activity of VPA on reporter gene activity (Kugler et al., 2015). Although the reduction of reporter protein activity correlates with impaired cardiac differentiation, future studies might be useful to investigate earlier time-points to evaluate whether an induction of the reporter gene can be found at lower concentrations. Interestingly, VPA, LiCl, and RA were also tested in the ReProGlo assay analyzing induction of a β-catenin inducible reporter in undifferentiated ESCs after 24 h of treatment (Uibel et al., 2010). Despite the experimental differences all three substances induced the reporter protein in the ReProGlo assay, although at distinct concentrations (see Table 1). In contrast to the ReProGlo and the BRE-ESC assays the BAT-Gal-ESC system introduced here is suitable to detect stimulatory and inhibitory activities. This seems particularly relevant for substances like LiCl that apparently can have both activities dependent on the differentiation status of the cells (Fig. 6).

Additionally we tested LDN193189, a specific Bmp inhibitor which prevents formation of the endodermal lineage (Kugler et al., 2015). BMP signaling is also highly conserved in evolution and cross-talk with Wnt signals has been described for vertebrate and invertebrate species under various physiological and pathological conditions (Itasaki and Hoppler, 2010). LDN193189 markedly reduced the reporter protein activity at similar concentrations as described for the BRE-ESCs and that correlate with its inhibitory activity on cardiac differentiation (Fig. 6). Thus, we cannot exclude indirect effects of substances on the reporter gene activity. However, these results also imply a much broader applicability domain of the BAT-Gal-ESC test system for the detection of teratogenic substances such as indirect effects on Wnt/β-Catenin signaling are also indicative for differentiation inhibiting mechanisms.

DPH and AA were used as negative controls, such that the general cytotoxicity of these substances directly affects differentiating cells rather than alterations in the differentiation process. Here we can only see changes in the reporter protein activity at concentrations markedly reducing the protein content of the lysates (Fig. 6, S1). Interestingly, the same holds true for 6-AN, which has been reported as a weak teratogen in several species through the inhibition of the pentose phosphate pathway (Honda et al., 1982). With the BAT-Gal-ESCs it was not possible to distinguish the effects on Wnt/β-Catenin activity from cytotoxic effects. Yet, we could previously show a clear reduction of Bmp signaling activity for 6-AN (Kugler et al., 2015). In light of the complexity of the earliest developmental processes there might not be one assay to predict teratogenic activities of substances *in vitro* (Adler et al., 2011). With the slight modifications of the EST by cultivating the EBs after three days on cell culture dishes and the *in vivo* compatible fluorophore C_{12}-FDG, the BAT-Gal-ESCs might be established as high throughput and high-content assay and included in an integrated testing strategy. Such a strategy might also include the recently described BRE-ESCs, since the BMP and Wnt/β-Catenin pathway are both highly conserved, involved in various developmental processes, and interact with each other. In addition, our data indicate that the two reporters do display distinct and specific reactions on known teratogenic substances. Thus, a combination is likely to generate complementary data and will help to elucidate the underlying molecular mechanisms of chemically induced teratogenicity.

Acknowledgement

We thank Stefano Piccolo and Tito Panciera for providing the transgenic mice and Riana Vogt (MPI-IE) for the generation of the BAT-Gal-ESC line. This work was supported by a grant of the FAZIT Stiftung to JK. Financial support via intramural funding of the BfR (SFP No. 1322-422 and 1322-497) is also gratefully acknowledged.

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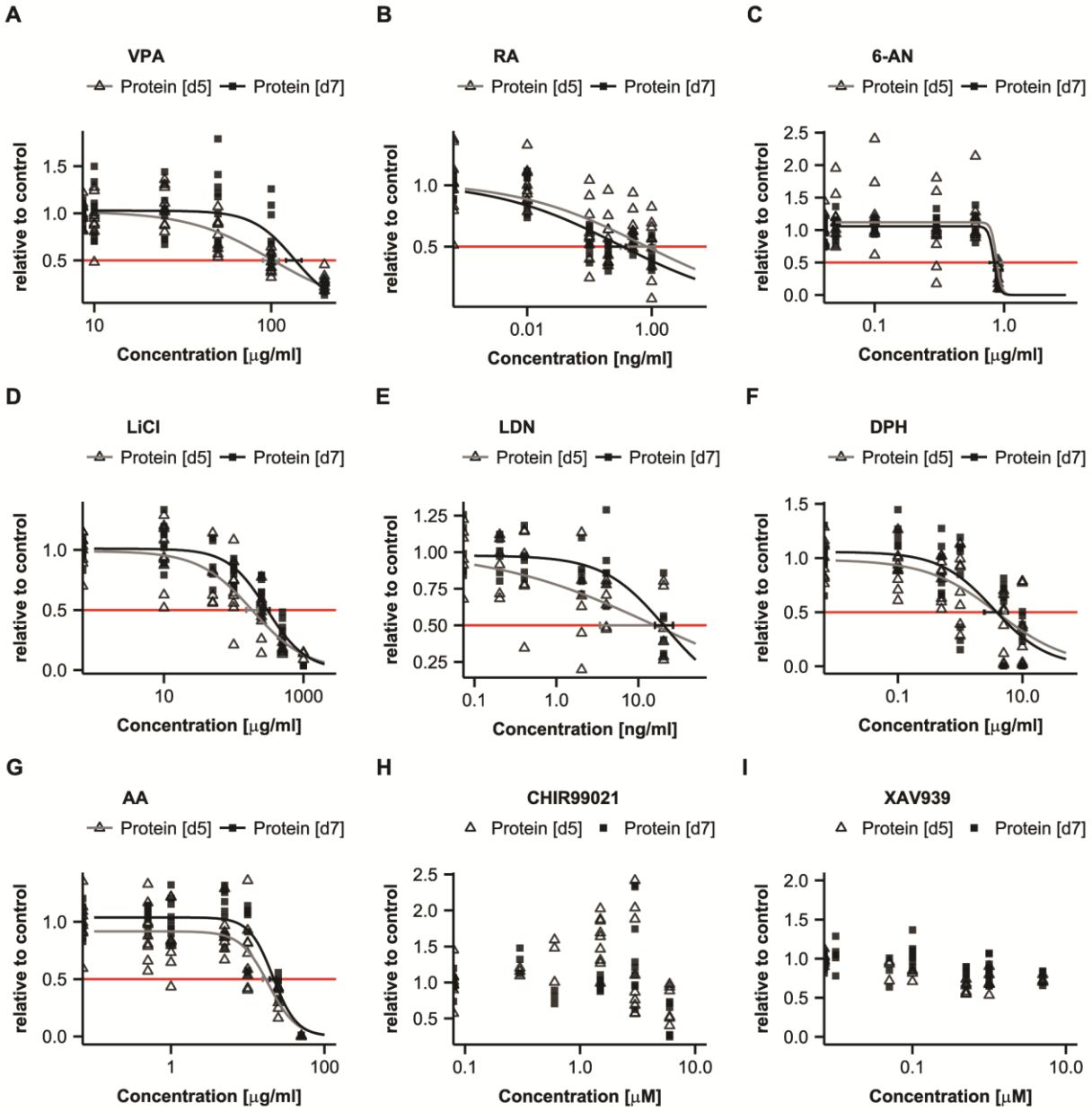
Supplementary

Supplementary Table 1: List of primers used for qPCR

Gene Name	Forward	Reverse
Gapdh	AGTGCCAGCCTCGTCCCGTA	CAGGCGCCCAATACGGCCAA
Ppia	CGCGTCTCCTTCGAGCTGTTTG	TGTAAAGTCACCACCCTGGCACAT
Pgk1	CTGACTTTGGACAAGCTGGACT	GCAGCCTTGATCCTTTGGTTG
LacZ	GCACCTCGCGGAAACCGACA	GGGTGAAACGCAGGTCGCCA
Brachyury	ATGCTGCAGTCCCATGATAAC	TGCGTCAGTGGTGTGTAATGT
Gata4	GGAAGACACCCCAATCTCG	CATGGCCCCACAATTGAC
Alcam	CAGTGGGAGCGTCATAAACC	CCTCAGGGGAAATGATAATTTACTA
Afp	GGCCGACATTTTCATTGGACAT	TGGGGGAGGGGCATAGGTTTT
Snail	CCCTTCAGGCCACCTTCTTT	GTCCAGTAACCACCCTGCTG
Map2	GGTTCCAAGGATAACATCAAACA	CATTTGGATGTCACATGGCTTA
Oct4	GGAGGGATGGCATACTGTGGACCT	AGCTCCTTCTGCAGGGCTTTCA



Supplementary 1: Dose-response curves of all tested substances on the protein contents of analyzed samples on day 5 and day 7, respectively.

3.3 Transgenic mouse models transferred into the test tube: New perspectives for toxicity testing in vitro?

With the BRE-ESCs and the BAT-Gal-ESCs I could show that the simultaneous evaluation of effects on the cardiomyocytes differentiation and reporter gene constructs for essential signaling cascades can increase the predictivity, shorten the assay duration, and yield information about the mode of action of a test substance. In the last years, numerous new transgenic mouse lines have been established and many of these express easy detectable reporter genes under the control of highly conserved, relevant signaling pathways. I propose that the combination of toxicological *in vitro* methods applying cells derived from these mouse lines provides a powerful combination of the advantages of both research areas: The *in vivo* verified biological relevance of transgene expression at least reduces the risk of integration position artifacts and the *in vitro* results can directly be compared and, thus, verified *in vivo*. Additionally, already established toxicological test systems, like the EST, can easily be adapted to the new cell lines and developed into high-throughput and high-content compatible test systems, increasing the efficiency and extending the possibilities to generate a large amount of information from a large number of potentially relevant chemicals in a short period of time.

The author's contribution:

Preparation of manuscript, table, and figure

Transgenic Mouse Models Transferred into the Test Tube: New Perspectives for Developmental Toxicity Testing In Vitro?

Kugler, Josephine, Andreas Luch, and Michael Oelgeschläger.

Trends in Pharmacological Sciences 2016; 37 (10). Elsevier Ltd: 822–30

doi:10.1016/j.tips.2016.06.009.

Online available at

http://dx.doi.org/10.1016/j.tips.2016.06.009.

Transgenic mouse models transferred into the test tube: New perspectives for developmental toxicity testing in vitro?

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Key Words: Teratogens, transgenic mouse models, *in vitro* testing, ESC, iPSC

Abstract:

Despite our increasing understanding of molecular mechanisms controlling embryogenesis, the identification and characterization of teratogenic substances still heavily relies on animal testing. Embryonic development depends on cell autonomous and non-autonomous processes including spatiotemporally regulated extracellular signaling activities. These have been elucidated in transgenic mouse models harboring easily detectable reporter genes under the control of evolutionarily conserved signaling cascades. We propose to combine these transgenic mouse models and cells derived thereof with existing alternative toxicological testing strategies. This would enable the plausibility of *in vitro* data to be verified in light of *in vivo* data and, ultimately facilitate regulatory acceptance of *in vitro* test methods.

Trends

Transgenic mouse models are ubiquitously used to understand signaling events during development and modern reporter gene approaches enable *in vivo* detection and quantification.

Disturbances during developmental processes or cell differentiation cause spatiotemporal alterations of signaling events, which therefore can be used to predict teratogenic effects of substances.

Utilizing recent advantages in embryonic and induced pluripotent stem cell generation and cultivation, transgenic cells carrying *in vivo*-characterized reporter constructs can be produced.

A plethora of *in vitro* assays has been developed for toxicological testing and can be advanced using the aforementioned transgenic cells to increase the information content and relevance of *in vitro*.

Developmental toxicity testing strategies

A total of 11 million animals were used in Europe in 2011 for experimental purposes, including 1 million of animals for toxicological risk assessment [1]. Most of the animals are applied in biomedical research and a significant number can be attributed to the development and use of transgenic animals that play a pivotal role in the characterization of human diseases [2]. In addition, it is expected that current chemical legislation in Europe (i.e., REACH) might lead to a significant increase in animal experimentation with estimates ranging from 4 to up to 54 million additional vertebrates needed [3, 4]. The testing for teratogenic activities according to OECD guidelines, in particular the prenatal development toxicity assay [5], the reproduction/developmental toxicity screening test [6], and the combined repeated dose toxicity assay with the reproduction/developmental toxicity screening test [7], already require high numbers of animals. Accordingly, the analysis of potential developmental toxicity makes up over 40% of all testing proposals submitted to the European Chemicals Agency by 2013 [8].

In the last years different approaches have been developed to address embryonic toxicity *in vitro*. Already in 2004 the murine embryonic stem cell test (EST) was validated by the European Center for the Validation of Alternative Methods (ECVAM) [9]. The EST uses the following three independent end-points to classify chemical substances: (1) the IC50 for somatic 3T3 cells, (2) the IC50 for embryonic D3 cells, and (3) the ID50 for the inhibition of the capacity of D3 cells to differentiate spontaneously into beating cardiomyocytes [10]. However, more cell types of the developing embryo actually need to be addressed [11] and therefore, protocols for the differentiation of embryonic stem cells into neurons [12], bone cells [13, 14], or endothelial cells [15] have been developed.

In parallel, the ToxCast program of the USA Environmental Protection Agency (EPA) [16] and the Tox21 consortium [17] focus on high-throughput and high-content analysis of multiple *in vitro* assays with diverse end-points to predict the *in vivo* toxicity of the chemical compounds under consideration. Thousands of chemicals are evaluated in hundreds of assays to generate toxicity profiles and to identify molecular and cellular pathways that mediate toxicity [18]. Using these data, Kleinstreuer *et al.* [19] were able to develop an *in silico* model to predict the chemical's impact on vascular development. Establishing the zebrafish as a model for developmental toxicity testing, one strategy uses phenotypic analysis of maturing embryos by utilizing high-content imaging methods [20, 21]. In a second approach, modern sequencing and microarray methods are used to gather mechanistic information that need to be analyzed and correlated with phenotypes [22] to identify toxicity pathways mediated by the tested compounds. These approaches are likely to be instrumental in the future to predict teratogenic activities of chemicals.

Processes during development and differentiation are orchestrated by few highly conserved, essential cellular signaling cascades and minute alterations may have strong impacts on the developing organism. The underlying mechanisms have been well characterized and especially transgenic mouse models have been used to uncover signaling activities *in vivo*. We could recently show that teratogenic effects can be monitored *in vitro* when analyzing reporter gene activities that represent specific cellular signaling pathways [23, 24]. Here we elaborate on the future potential to combine toxicological testing methods with transgenic mouse models and cells derived thereof (Figure 1).

Transgenic mice—General aspects and toxicity testing

In basic and biomedical research high-content and high-throughput approaches already play an important role in the characterization of biological processes and the detection of druggable new lead structures [25–27]. Since these methods are particularly suited to unravel molecular mechanisms and regulatory networks that mediate physiological, pathological, or toxicological processes, they also entered the field of pharmacology and toxicology [28]. Despite these developments, our knowledge on molecular mechanisms and biological functions, including embryonic development, is still highly dependent on the studying of genetically modified mouse models. Ever since the dawn of modern molecular techniques that enabled the generation of transgenic mice by pronuclear injection [29] and specific genomic manipulations by homologous recombination [30], thousands of mouse strains have been generated that either mimic specific aspects of human diseases or that facilitate the analysis of the function of single genes as well as complex signaling pathways. With the more recent development of the CRISPR/Cas technique, a new area might already have begun with an enormous additional number of modified mouse lines to be expected in the years ahead [31]. The Jackson Lab, that supplies the biggest compilation of transgenic animal models, already comprises over 7000 different lines ranging from humanized mouse models over diverse disease models. In contrast to the importance and widespread use of genetically modified mouse strains, there are only a few of them actually applied in the toxicological assessment of chemicals [32]. Up to date only the transgenic rodent mutation assay created to detect chemical mutagens [33] has received regulatory acceptance. Another well-established, although not validated, mouse model expresses luciferase under the control of an estrogen response element (ERE) promoter, thereby allowing the investigation of estrogen receptor-dependent transcriptional activity *in vivo* through optical imaging in living animals [34]. This mouse model enables the quantification and specific profiling of the acute or prolonged endocrine disruptor activity of suspected compounds *in vivo* [35, 36], thereby proving the strengths and advantages of transgenic models over conventional animal testing.

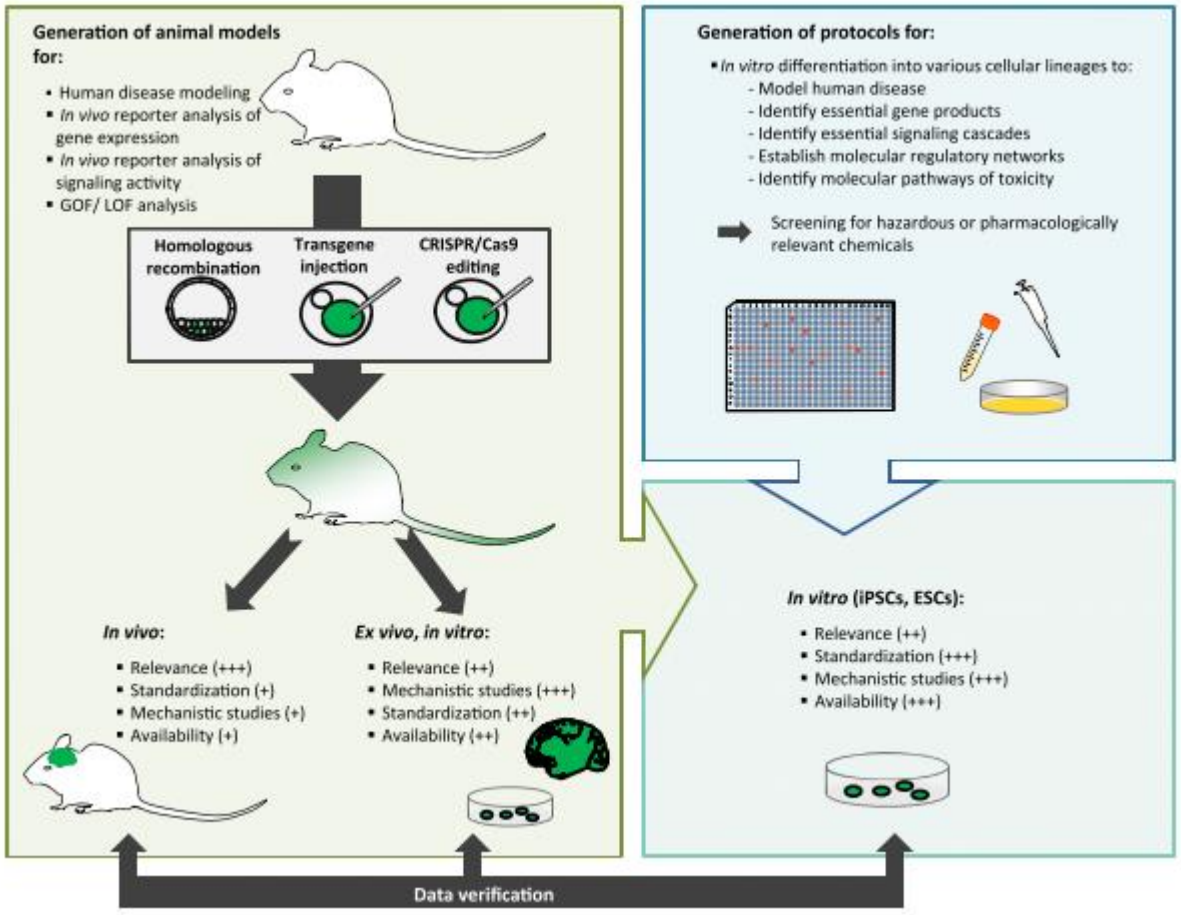

Figure 1. Combination of transgenic mouse models and existing *in vitro* methods. In basic research transgenic animal models are generated as human disease models, to study gain-of-function (GOF) and loss-of-function (LOF) of specific genes but also to analyze the expression of reporter genes as read-out for gene expression and signaling activities *in vivo*. With these models *in vivo, ex vivo*, and *in vitro* experiments are possible. On the other side, a plethora of protocols for the differentiation of cells into various lineages are available and used for toxicological studies, including high-content and high-throughput screening. We propose to use the *in vivo* characterized constructs to generate ESCs or iPSCs in combination with existing protocols for toxicological testing to raise the relevance of the afterwards results obtained at once with the possibility to gain additional mechanistic information on molecular pathogenesis.

Evolutionary conserved signaling cascades in embryonic development and toxicology

Transgenic mouse models harboring a suitable reporter gene under the control of an essential signaling cascade allow a direct measurement and quantification of signaling activities during embryogenesis, organogenesis, or adult tissue homeostasis within the living animal in real time. To assess chemical-induced perturbations, we suggest cellular signaling cascades to be a great possibility to gather in-depth information on the mode of action and the underlying molecular mechanisms of tested substances also *in vitro*. Intriguingly, organogenesis and embryogenesis, but also cell differentiation in adult organisms, are

orchestrated by a limited number of essential signaling cascades. These are highly evolutionarily conserved from fly to man and include receptor tyrosine kinases (RTK), Wnt, TGF-ß, Shh, Notch and nuclear receptors, such as retinoic acid or steroid hormone receptors [37,38]. These signaling pathways do not only display highly conserved functions during cellular differentiation, processes during embryogenesis, and adult tissue homeostasis but have also been implicated in the development of human diseases, including cancer [39–41].

Table: 1: Compilation of transgenic mouse models expressing a reporter gene under the control of a particular cellular signaling cascade.

Signaling Pathway	Expression *in vivo*	Construct and reporter gene	Source
AR	Embryo: testis, skin, brain Adult: bone marrow, brain, epididymis, fallopian tube and oviduct, ovary, prostate, salivary gland, seminal vesicle, spleen	AR promoter controlling expression of AR fusion protein with Gal4-DBD controlling expression of luciferase with a Gal4-promoter	[58]
		SC1.2-ARE with Tk minimal promoter fused to Luc-hPEST	[59]
RA	Embryo: inner cell mass and trophectoderm; primitive streak and posterior region; mid region and developing eye	3xRA (RAR beta2) with promoter of Hsp68 control expression of LacZ	[60]
		3xRA (RAR beta2) with Tk minimal promoter control expression of LacZ	[61]
Notch	Embryo: epiblast, neural plate, cardiac region, vasculature Adult: lung, heart, kidney, spleen, testis, thymus	CBF1-RE fused to SV40 minimal reporter control H2B-Venus	[62]
ER	Adult: uterus, pituitary, hypothalamus, kidney, liver, adrenal and thyroid gland, bone, brain, tongue, liver, mammary gland	3xERE (vitogellin ERE) fused to TK promoter control LacZ-SV40 polyadenylase	[63]
		2xERE fused to TK promoter control luciferase expression, construct flanked by insulator Hs4	[64]

Wnt	Embryo: posterior epiblast, primitive streak, mid- and hindbrain, neural crest cells, heart anlagen, tail bud, newly formed somites, tips of outgrowing limbs, otic vesicles; first and second branchial arches, nasal fold, apical ectodermal ridge, mammary buds, periocular tissues; kidney Adult: only few cells: liver - hepatocytes around the central vein, activated T cells through liver injury, positive cells in spleen	6x Tcf/Lef enhancer elements fused to Tk promoter control expression of eGFP/LacZ; construct flanked by insulator Hs4	[65]
		7xTcf/Lef binding sites fused to minimal promoter of siamosis gene control expression of LacZ	[54]
		3xTcf/Lef consensus sequence fused to c-fos minimal promoter control expression of LacZ	[66]
		7xLef1 binding sites fused to c-fos minimal promoter control expression of eGFP	[67]
		5.6kb of Axin2 promoter, exon 1 and intron 1 control expression of LacZ/ eGFP	[68]
Shh	Embryo: neural tube, myotomal cells at thoracic level for differentiation into myocytes	8x Gli-binding sites fused to hsp68 minimal promoter control expression of eGFP	[69, 70]
Bmp	Embryo: posterior primitive streak, heart region, kidney, bone, liver, pancreas, lung, hair follicle, central nervous system, and vasculature Adult: vasculature, large airways, alveoli, liver, pancreas, hair follicle	Id1-BRE upstream of Hsp68 promoter control expression of LacZ followed by SV40 polyadenylation signal	[71]
		7x Id3-BRE with minimal promoter of Id3 control expression of LacZ or luciferase	[72]
		CMV enhancer fused to 2xId1-BRE and MLP minimal promoter control expression of eGFP or LacZ	[51, 73]
TGF-β	Adult: brain (mainly neurons), intestine (Peyer's patches), skin, heart	12xSBE fused to Tk minimal reporter control expression of luciferase and SV40 late polyadenylation signal	[74]

NF-κB	Embryo: cerebellar and the olivary nuclei in the rhombencephalon, spinal medulla, thymus, weak in epithalamus, blood vessels Adult: thymus and bone marrow, histiocytes, endothelial cells, lymph nodes, spleen, Peyer's patches, brain (cortex, cerebellum: neurons)	3.1 kb of p105 promoter / three copies of NF-κB-BD site of immunoglobulin κ light chain enhancer upstream of conalbumin minimal promoter control expression of LacZ with NLS and SV40 adenylation signal	[75]
		three copies of NF-κB-BD site of immunoglobulin kappa light chain enhancer upstream of conalbumin minimal promoter control expression of Luciferase	[76]

Abbreviations: AR – androgen receptor; ARE – androgen response element; Bmp – bone morphogenetic protein; CBF1 - recombination signal binding protein for immunoglobulin kappa J region; CMV – cytomegalovirus; DBD – DNA binding site; eGFP – enhanced green fluorescent protein; ERE – estrogen response element; Hs4 – β-globin hypersensitive site; Hsp68 – heat shock protein 68; ICM – inner cell mass; Lef – leukemia inhibitory factor; Luc – luciferase; MLP – major late protein; NF-κB – nuclear factor κB; RA – retinoid acid; RAR – RA receptor; RE – response element; SBE – binding site for Smad3/4; SC1.2 - prostate transepithelial transporter IgM; Shh – sonic hedgehog; SV40 – Simian virus 40; Tcf – transcription factor; TGF-β – transforming growth factor β; Tk – thymidine kinase.

A large amount of existing and well characterized transgenic mouse models that allow the *in vivo* analysis of most of these pathways have already been established (some of them are compiled in Table 1). However, given the large number of chemicals that need to be assessed, it seems neither feasible nor compatible with animal welfare considerations to directly introduce transgenic models into regulatory toxicology and risk assessment procedures [42]. Instead, transgenic reporter cell lines have been developed that also allow high-throughput screening for specific activities. These include, amongst others, cell lines harboring reporter genes under the control of ERE promoters [43] that enable identification of endocrine active substances, as well as embryonic stem cells carrying a reporter gene under the control of the canonical Wnt signaling pathway that will be instrumental in the identification and analysis of teratogenic compounds [44]. However, the expression of the respective reporter genes do not necessarily reflect endogenous regulation of target genes *in vivo* that can highly depend on promoter composition and the availability of essential and target gene specific co-factors [45–47]. In addition, the random integration of transgenes is highly prone to significant influences of surrounding genomic sequences on reporter gene expression, as such position effects have been shown to be rather common [48].

Combination of *in vitro* methods with mouse models

To maximize the possibilities and utilization of both the current *in vitro* approaches and the *in vivo* models on hand, one can combine the advantages of transgenic cell lines, e.g., the easy handling, cost-effectiveness and high-throughput compatibility, with the advantages of transgenic animals that ultimately enable the proof of biological relevance. In the frame of this concept, the latter will be used as source and for the generation of cell lines, as for example embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), or primary cells. This ensures that the transgene actually reflects biologically relevant processes and allows high-throughput/high-content approaches to be applied (Figure 1). Most importantly, it also might help to minimize the use of live animals in toxicological testing, drug screening, or profiling as well as in basic biomedical research. A comparable approach has been already introduced on the human side with the use of patient-derived iPSCs for the analysis of complex human diseases in vitro [49, 50].

We have recently described an ESC line harboring an enhanced GFP reporter under the control of a Bmp responsive promoter to be applied in an assay aimed at detecting potentially teratogenic chemicals [23]. *In vivo* the GFP reporter gene is expressed in the cardiovascular system and several other organs later on, as expected in light of the already known importance of Bmp signaling for the differentiation of various tissues during embryonic development [51]. *The in vivo* analysis of the transgene ensured that the promoter indeed recapitulates endogenous Bmp signaling activity and excluded at least to a large extent potential position effects. Importantly, the analysis of the ESC line proved the in vivo reporter gene activity being mirrored in vitro. During the differentiation of ESCs into cardiomyocytes, the endogenous induction of the Bmp reporter takes place between day 5 and day 7 of differentiation. The analysis of GFP-positive cells revealed the endothelial and endodermal descent of cells. In the developing heart *in vivo* endodermal cells in proximity to mesodermal cells have been shown to induce differentiation of the latter into cardiac mesoderm [52]. Accordingly, a similar pivotal role of endodermal cells in cardiac specification has also been demonstrated *in vitro* [53]. Upon treatment with chemical teratogens, these cardiac differentiation processes become disturbed, an effect that can be detected and monitored via reporter gene expression. To this end, decreasing overall GFP expression indicates and correlates to the functional loss of beating cardiomyocytes. Additionally, some substances alter the cell fate, which can be judged via microscopic evaluation of GFP-positive cells, changing the former net-like structure of endothelial cells into clustering GFP-positive cell aggregates within the outgrowths of the embryoid bodies [23]. In general, the quantitative read-out of the transgene indicates hampered differentiation while qualitative analysis of the GFP-positive cells can be used to detect altered cell fate, both of which aspects making the assay high-

content and high-throughput compatible. In future studies this test system might be transferred also to a row of other established differentiation protocols, as for example neurons, bone, and vasculature, thereby enabling the analysis and characterization of both the role of Bmp signaling in other cell types and the kinds of alterations induced by teratogenic compounds.

Similarly, we were able to show in a recent comparable study using another murine ESC line with a reporter indicating active Wnt/β-catenin signaling that teratogenic activities of substances can be monitored using this endpoint as well [24]. This reporter was expressed *in vivo* in the primitive streak and consecutively mesodermal cells throughout development faithfully recapitulated known Wnt/β-catenin signaling centers [54]. The characterization of reporter gene positive cells *in vitro* confirmed that these cells indeed are of mesodermal origin and express genes involved in epithelial to mesenchymal transition [55]. During heart development and cardiomyocyte differentiation Wnt/β-catenin signaling has a biphasic role and the same could be seen in our *in vitro* system: whereas Wnt/β-catenin signaling has to be induced for mesoderm formation, the following cardiomyocyte specification is dependent on reduced levels of Wnt/β-catenin signaling [56]. Intriguingly, using various model substances for teratogenicity, induction as well as reduction of the reporter could be associated with compound's teratogenic activity in a time-dependent manner [24].

In a broader perspective, in addition to ESCs also iPSCs and primary cell cultures as well as 3D organotypic tissues generated of transgenic mouse models might become applicable to be tested against teratogenic and otherwise toxicologically relevant compounds. For developmental processes models reflecting signaling activities seem natural, but for other aspects other transgenic lines could be used as well. For drug safety testing, humanized or especially susceptible models could help to minimize negative side effects before clinical studies will begin. Moreover modern techniques enable the combinatorial use of more than one reporter gene, thereby allowing co-cultivation of different cell types. This would help recapitulating the complex *in vivo* situation in a much better way, but still allow dissecting the molecular interactions in an interpretable manner.

Concluding Remarks

Given the enormous number of transgenic mouse lines reporting on essential cellular signaling cascades we propose to take advantage of these models in toxicological test systems *in vitro*. With this approach toxicological research as well as regulatory needs in the safety assessment of chemicals would be well supported and benefited by means of basic or biomedical research.

There are various attempts to optimize toxicological testing using human cell lines, including ESCs or iPSCs. Certainly, these cells have a great potential enabling to address all kinds of basic biological functions at the molecular level. However, while being instrumental as tools in regenerative medicine and the molecular analysis of human diseases, an appropriate *in vivo* verification of relevant reporter gene expression is usually excluded. Further, reliable human reference data is scarce, in particular with respect to developmental toxicity. For these reasons, new test methods for chemicals are commonly being validated against data from rodents (rat or mouse), although it has been shown that it rather reveals difficult to demonstrate that an *in vitro* test appropriately reflects human hazards [57].

Still numerous questions remain open and are beyond the scope of this article (see Outstanding Questions box). Nevertheless, our examples demonstrate that modern transgenic systems combined with established toxicological test systems broadens the usability and adds information on the possible mode of action of the tested substances, thereby helping to minimize the need for experimental live animal testing in regulatory pharmacology and toxicology in the future.

Outstanding Questions

How can *in vitro* differentiation and substance testing be combined with high-throughput and high-content analyses?

How can intellectual property issues associated with the use of transgenic mouse models and cells derived thereof be solved to facilitate their use in toxicological testing?

How can we further accelerate the regulatory acceptance of in vitro test systems in light of the steadily increasing numbers of experimental animals used?

Should cells from humanized mouse models be used to facilitate regulatory acceptance?

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4. Discussion

Until today the assessment of the teratogenic potential of substances like drugs or chemicals relies on animal testing. With the already discussed changes in legislation the need for *in vitro* alternatives becomes more pressing. The main focus of this work was the evaluation whether highly conserved and essential signaling cascades are suitable as a read out to predict a teratogenic potential of the analyzed substances and whether additional conclusions on the mode of action can be drawn. In the sections 3.1 and 3.2 two different cell systems have been analyzed and established as predictive tools *in vitro*.

The first signaling pathway I worked with was the Bmp signaling cascade: the BRE-ESCs were generated from a transgenic mouse line described by Monteiro *et al.* (2008) in which the reporter gene EGFP was shown to be expressed in known Bmp activity centers during embryonic development. Thus the mouse line proved a reliable tool *in vivo* and illustrated the versatile role of Bmp signaling in the formation of various organs throughout development (Zhao 2003). Similarly, I could show that the BRE-ESCs, mouse embryonic stem cells derived of the transgenic BRE mouse line, reliably differentiated into cardiomyocytes within ten days and exhibit a comparable differentiation kinetic as the previously described D3-ESCs within the embryonic stem cell test (EST, Genschow et al. 2004). EGFP expression could already be detected in the undifferentiated, pluripotent ESCs, but increased during differentiation and nicely correlated with the phosphorylation of intracellular signaling mediators, like Smad1, and the expression of known direct target genes, like Id genes. The induction of Bmp activity took place about day 5 of differentiation and peaked at day 7, therefore nicely correlating with mesoderm induction (Winnier et al. 1995).

The easily detectable EGFP allows the morphological analysis of reporter gene positive cells in living tissues without destructive methods. In our system we could identify two different cell morphologies: (1) cells forming a net-like structure and (2) small cells distributed over the entire outgrowth of the EB, but especially being in the vicinity of the beating cardiomyocytes. Interestingly, none of the beating cells itself were positive for the reporter gene. These observations were in accordance with the results of marker gene expression in sorted cells showing that the EGFP positive cells specifically express marker genes for endothelial and endodermal cells.

The differentiation into endothelial cells had already been used to predict teratogens *in vitro* (Festag et al. 2007a, 2007b) and, interestingly, the ToxCast data had been used to predict teratogenic activity of compounds in an *in situ* approach using endpoints reflecting endothelial differentiation and vascular development (Kleinstreuer et al. 2011). Taken together the functional differentiation of endothelial cells is essential for correct development and the BRE-ESCs might be used to further explore this aspect as the

reporter gene allows the morphological evaluation of vascular networks in living tissues, maybe even in a high-content, high-throughput approach.

I investigated the specificity of the reporter gene construct *in vitro* using LDN193189 and SB431542 two specific inhibitors for Bmp and Tgfβ signaling, respectively. Although both inhibitors markedly impaired cardiac differentiation only LDN193189 reduced the EGFP expression. Upon further characterization of the effect of LDN193189 during the differentiation, it could be seen that the inhibition of Bmp signaling induced formation of ectodermal tissues in lieu of endoderm and mesoderm. *In vivo* the underlying endodermal cell layer induces the specification of cardiac mesoderm through expression of Bmp (Van Vliet et al. 2012) and *in vitro* it was shown that Sox17, an endodermal marker, is essential for the cardiomyocyte differentiation as well (Liu et al. 2007). Together with the observation of EGFP positive cells in the vicinity of beating foci and the endodermal cell fate of EGFP positive cells, it would be interesting to elucidate whether these cells express Bmp in order to induce the cardiac specification. I could further narrow down the time window in which LDN193189 effectively prevented Bmp activity. A treatment from day 3 to day 4 of differentiation, the time of mesoderm induction, was sufficient to impair cardiac differentiation and to reduce EGFP expression. This short effective time frame in which a substance needs to be applied is explainable with the multi-step process of cardiac differentiation and the notion that every organ has its own susceptible time window for teratogens (DeSesso 2010).

Fitting these observations, the Wnt inducer CHIR99021 reduced EGFP expression and impaired cardiac differentiation when applied throughout differentiation, but changed the morphology of the EGFP positive cells when applied from day 3 of differentiation onward. Under this treatment regime we could observe clusters of EGFP positive cells indicating a cell fate switch and once more hinting to the high-content compatibility of our test system, as not only loss of EGFP can be correlated with impaired differentiation but also morphological changes in EGFP positive cells that can be detected and quantified with suitable bio-imaging systems.

Even more pronounced is the time dependence within the BAT-Gal-ESC system. The onset of reporter gene expression correlated nicely with the direct Wnt/β-Catenin target gene Brachyury, as marker for mesoderm induction *in vitro* and *in vivo* (Arnold et al. 2000; Kispert and Herrmann 1994). Wnt/β-Catenin signaling has a biphasic role during cardiomyocyte differentiation: it has to be induced during mesoderm induction, while subsequent downregulation is favorable for cardiac specification (Ueno et al. 2007). This is also nicely recapitulated in the BAT-Gal mouse line, showing a faint reporter gene expression in the early heart anlagen, with no staining on the later course of development (Maretto et al. 2003). The identification of the reporter gene positive cells revealed that mainly mesodermal cells are under the influence of

canonical Wnt signaling and, additionally, cells undergoing epithelial to mesenchymal transition (EMT), a morphological and functional change within cells essential for development and differentiation *in vivo* and *in vitro* (Kim et al. 2014; Lim and Thiery 2012; von Gise and Pu 2012). The differentiation of the BAT-Gal-ESCs could be nicely compared with the BRE-ESCs, although it was a bit delayed. In both systems the reporter gene expression was markedly induced upon mesoderm induction or more general during differentiation of the three germ layer, indicating overlapping as well as distinct functions for canonical Wnt and Bmp signaling. The correlation of the different reporter gene activities and mesoderm formation fits the expression and function of these two pathways *in vivo* and a lack of Bmp or deregulated (reduced or increased) Wnt activity is connected to failure in mesoderm formation. The delay in differentiation of the BAT-Gal-ESCs in comparison to BRE-ESCs could be due to the different mouse strains from which the ESCs were generated, although only the functional differentiation into beating cardiomyocytes was delayed whereas the transcriptional profile of the various marker genes during the differentiation process was rather comparable.

The biphasic role of canonical Wnt signaling could also be observed when treating the cells during the differentiation with specific inhibitors. XAV939 and CHIR99021 both alter the intracellular signaling cascade. While XAV939 inhibits the dissociation of the destruction complex upon Wnt stimulation thereby preventing the intracellular transmission of the signal (Huang et al. 2009), CHIR99021 inhibits Gsk3β and thus the destruction complex such that active Wnt signaling is mimicked (Bain et al. 2007). Both inhibitors impair the differentiation into cardiomyocytes, but also have specific effects. CHIR99021 induces canonical Wnt signaling und increases the reporter gene expression. Interestingly, the effect cannot further increase the endogenous induction during normal differentiation. With XAV939, the inhibition of canonical Wnt signaling can only be seen from day 5 of differentiation on, when the normal induction takes place. These results led to the assessment of two different time points to be able to see an induction as well as a reduction of the reporter gene expression which both could be correlated with impaired differentiation.

For the BAT-Gal-ESCs we analyzed the effect of non-teratogenic compounds, diphenhydramine and acrylamide. As expected, effects on reporter gene expression was only seen at concentrations that reduced the protein content of the samples below 50 %, an indicator for general cytotoxic effects. Additionally, LiCl was analyzed as weak teratogen and as positive control substance for Wnt induction (Klein and Melton 1996; Wexler et al. 2008). The reporter gene expression was induced on day 5 of differentiation, but also reduced on day 7 which well correlated with the impaired cardiac differentiation and suits the *in vivo* findings of LiCl being implicated in cardiac malformations (Weinstein and Goldfield 1975).

Valproic acid (VPA) is a histone deacetylase inhibitor (HDACi) through which its effects are conveyed (Wiltse 2005). In other cell systems the inhibition of HDAC raises the β-Catenin protein level, thereby increasing canonical Wnt signaling. We could only detect an increase in reporter gene expression in the BAT-Gal-ESCs at day 5 of differentiation at concentrations showing cytotoxic effects as well. On day 7, the reporter gene activity was lowered correlating with the impaired cardiac differentiation. Interestingly, the EGFP expression in the BRE-ESCs was as well lowered. Hezroni *et al.* (2011) suggested that the HDACi effect of comparable concentrations of VPA induces pluripotency, which is in line with the use of VPA in the generation of induced pluripotent stem cells (iPSCs) from mouse and human cells (Romito and Cobellis 2016). The notion that VPA increases or sustains the pluripotent character of the ESCs would explain that VPA had to be applied from the beginning of the differentiation, adding VPA on day 3 or later did not interfere with the cardiac differentiation or expression of EGFP in the BRE-ESCs anymore. Thus I would conclude that the overall inhibitory effect of VPA on differentiation is more relevant for its teratogenic activity than the potential stimulatory activity on canonical Wnt signaling.

The same time dependency was seen for retinoic acid (RA, not published). Although the mechanisms through which RA functions are completely different, it had to be applied from day 0 onward. This might be explained with the fact that RA, as natural occurring morphogen, is generated *in vivo* at embryonic day 8 p.c. (Balkan et al. 1992; Rossant et al. 1991), which can be compared with day 4 or 5 of differentiating ESCs. There are several publication describing the neural inductive potential of RA *in vitro*, although using rather high concentrations (10^{-8} – 10^{-6} M instead of 10^{-10} M in our study) from day 2 of differentiation on (Okada et al. 2004), as well as the cardiac differentiation promoting effect of RA in comparable concentrations, but only from day 5 onward (Wobus et al. 1997). Early exposure to the teratogen alters the differentiation program *in vitro*, thus inhibiting the normal process. Interestingly, Kennedy et al. (2009) analyzed the effect of RA on differentiating ECSs from day 0 on and could show, that RA activates the expression of Wnt3A, a mesodermal marker, and thus activates canonical Wnt signaling. Additionally, they could show that RA inhibits Bmp signaling and overall promotes the differentiation of skeletal muscle cells in lieu of cardiomyocytes. This is in accordance with the results obtains in my studies, RA reduced the activity of Bmp signaling in the BRE-ESCs and markedly increased canonical Wnt signaling in the BAT-Gal-ESCs with concordant inhibition of cardiac differentiation.

The teratogenic potential of 6-aminonicotinamide (6-AN) was harder to detect in our systems: Through the inhibition of the pentose phosphate pathway which produces diverse substrates for the synthesis of biomolecule such as nucleotides and aromatic acids, fast growing and dividing systems, like the developing embryo or differentiating cells, are especially susceptible to the toxic effect of 6-AN. In the BAT-Gal-ESCs

system we could not detect any specific effect, but could show that it inhibits the differentiation. However, a reduction of Bmp activity could be detected in the BRE-ESC, maybe reflecting a secondary effect of 6-AN on the overall differentiation process. This result stresses the necessity to evaluate the teratogenic potential of a given substance in more than one *in vitro* assay.

The last aspect, I want to discuss is the cross talk of Bmp and Wnt signaling. As already mentioned CHIR99021 exhibited a time dependent reduction of the EGFP signal in BRE-ESCs and changed the morphology of the EGFP positive cells, when applied from day 3 onward. Thus it altered the cell fate of cells receiving a BMP signal and had an influence on Bmp signaling itself, too. Interestingly, XAV939 also reduced the Bmp activity and cardiac differentiation at all time frames tested indicating that increased as well as decreased Wnt signaling affects the endogenous Bmp activity during differentiation of the ESCs, but with different time dependencies. On the other side, LDN193189 nicely reduced canonical Wnt signaling as well as Bmp activity in correlation with the impaired cardiac differentiation. In the systems introduced, we are not able to dissect whether the observed effects are direct or indirect. The cross talk of Bmp and Wnt are still under investigation, but it could be shown, for example, that Gsk3β can also phosphorylate Smad1, thus inhibiting intracellular transmission of Bmp signaling, and that a non-canonical Bmp pathway can directly inhibit transcriptional activity of β-Catenin/Tcf complexes in the nucleus (Fuentealba et al. 2007; von Bubnoff and Cho 2001). These interactions together with the non-canonical pathways add to the complexity of the introduced signaling cascades and will be an interesting aim for further studies, in particular when their significance in development and differentiation is better illuminated.

5. Conclusion and Outlook

In the last chapter I discussed the results of the experimental part of my work. It was possible to establish two independent cellular assays that can be used to predict teratogenic activities of compounds *in vitro*. As we have seen, the differentiation into cardiomyocytes is a multi step process and is regulated by intracellular clues such as signaling cascades. The use of ECSs harboring reporter gene constructs which have previously been characterized *in vivo* reduces the risk of artefacts due to the integration site. Both signaling cascades, the Bmp and the Wnt/β-Catenin pathway, were endogenously induced during the differentiation and were effected by specific inhibitors and teratogenic substances. Therewith the consideration of using highly conserved and essential signaling cascades as read out for the teratogenic potential of a substance proved to be valuable, especially, as also mechanistic insights into the molecular mechanisms mediating the teratogenic activity of the model substances test could be drawn and verified by the data from recent literature.

In section 3.3, we reviewed the possible benefits of integrating the existing transgenic mouse lines with established toxicological test systems in detail. Our examples demonstrate that such a procedure can result in an advanced assay with more mechanistic information and the possibility of creating assays that are high-throughput and high-content compatible. Additionally, in the light of recent advances in genetic manipulation of cells the introduction of a second reporter gene would facilitate the simultaneous detection of, for example two signaling pathways in parallel or simplify the functional read out of such an assay.

My work only describes the first step of establishing these assays as a proof of concept study and further substance tests need to be performed and evaluated to validate the assays as *in vitro* alternatives.

6. Summary

The assessment of a teratogenic potential of substances and compounds currently relies on time and cost intensive *in vivo* tests, although recent changes in legislation increase the need for *in vitro* alternatives. My work is a proof of concept study, analyzing whether highly conserved, essential signaling cascades can be used as predictive tool for teratogenic activity. Signaling cascades regulate embryonic development *in vivo* as well as the differentiation of embryonic stem cells (ESCs) *in vitro* and the latter already had been shown to be suitable as a predictive tool in the embryonic stem cell test (EST). For my studies I focused on two different cascades, the canonical Wnt and Bmp signaling pathways. The used transgenic ESCs were generated from well described transgenic mouse lines, each harboring an easily detectable reporter gene under the control of one essential signaling pathway.

The canonical Wnt pathway is essential for the mesoderm induction and the reporter gene in the BAT-Gal-ESCs was expressed in mesodermal cells and cells undergoing epithelial to mesenchymal transition. During cardiac differentiation, the Wnt pathway has a biphasic role, which could be monitored *in vitro* as well. For the detection of an increase and/or a decrease in reporter gene activity, two independent time points were analyzed. Increased as well as decreased reporter gene activity was associated with impaired cardiac differentiation *in vitro* such that the BAT-Gal-ESCs could indeed be used as predictive tool for teratogenic activities.

The BRE-ESCs, expressing the green fluorescence protein (EGFP) under the control of Bmp signaling, showed an endogenous induction of EGFP during the differentiation into cardiomyocytes. Especially endodermal and epithelial cells expressed high amounts of EGFP and the endodermal cells proved to be essential for the specification of cardiac mesoderm. Upon treatment with model substances a dose-dependent decrease in EGFP expression and cardiomyocyte differentiation was detectable, demonstrating suitability of this cell line for the testing of teratogenic activities as well.

In general, the data strongly supports the working hypothesis that highly conserved, essential signaling cascades can be used to predict teratogenic substances during the differentiation of ESCs. And along with the general inhibition of differentiation, these cell systems provide mechanistic information and can even shorten assay duration.

7. Zusammenfassung

Während der Embryonalentwicklung entstehen aus einer befruchteten Eizelle sämtliche Gewebe, Organe und Strukturen des späteren Individuums. Die Komplexität des Vorganges bedingt, dass äußere Einflüsse dramatische Auswirkungen auf diesen streng regulierten Prozess haben können und die Identifizierung in dieser Weise wirkenden Substanzen im Besonderen erfolgt mit Hilfe von zeit- und kostenintensiven Tierversuchen. Verschiedene gesetzliche Änderungen der letzten Jahre haben dazu geführt, dass *in vitro* Alternativen zum Tierversuch eine zunehmenden Bedeutung erlangten. In dieser Arbeit sollte eine *in vitro* Alternative, der embryonale Stammzelltest (EST), zur Testung von Teratogenen im Hinblick auf neue Endpunkte weiter entwickelt werden. Der EST betrachtet die Differenzierung der ESCs in spontan schlagende Herzmuskelzellen. Dieser Vorgang ist ähnlich strikt reguliert wie die *in vivo* Entwicklung und häufig genutzt zur Aufklärung der zugrunde liegenden Prozesse *in vitro*. Dazu nutzte ich murine, embryonale Stammzellen (ESCs), die aus transgenen Mauslinien gewonnen wurden und ein leicht zu detektierendes Reportergen unter dem Einfluss von hoch konservierten, essentiellen Signalkaskaden besitzen. Dabei habe ich mich auf den Bmp und den Wnt/β-Catenin Signalweg konzentriert.

Die BRE-ESCs, die ein grün-fluoreszierendes Protein (EGFP) unter der Kontrolle des Bmp Signalweges exprimieren, zeigten eine endogene Induction der EGFP Expression während der Herzmuskeldifferenzierung. Die Analyse der EGFP-positiven Zellen ergab, dass es sich um endodermale und endotheliale Zellen handelt, wobei die endodermalen Zellen für die Spezifikation des Mesoderms in kardiogenes Mesoderm essentiell sind. Die Analyse teratogen wirkender Substanzen zeigte eine dosisabhängige Reduktion der EGFP Expression, die gut mit der Verminderung der Herzmuskeldifferenzierung korrelierte.

Das zweite Zellsystem, die BAT-Gal-ESCs, exprimieren ihr Reportergen unter dem Einfluss des kanonischen Wnt-Signalweges. Auch dieser Signalweg wird während der Differenzierung endogen induziert und ist eng mit der Mesoderminduktion assoziiert. Dies konnte ich bestätigen, da die reportergenpositiven Zellen mesodermalen Ursprungs waren. Zusätzlich zeigte sich, dass Zellen, die eine epithelial-mesenchymale Transition durchlaufen ebenfalls über eine erhöhte Aktivität des Wnt Signalweges verfügen. In diesem Testsystem konnte gezeigt werden, dass sowohl eine künstliche Induktion als auch eine Reduktion der Reportergenaktivität mit einer verminderten Herzmuskeldifferenzierung einhergehen kann und auch diese Zellen sich als prädiktives Testsystem eignen.

Damit konnte in der vorliegenden Arbeit die Arbeitshypothese bestätigt werden, dass eine Analyse von essentiellen, hoch konservierten Signalkaskaden sich für eine Vorhersage eines teratogenen Potentials einer Substanz eignen. Zusätzlich können durch die Erfassung mehrerer Endpunkte in einem Zellsystem auch mechanistische Informationen gewonnen werden, so dass eventuell die Wirkungsweise der verschiedenen, potentiell teratogenen Stoffe genauer charakterisiert werden kann.

8. Literature

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9. Abbreviations

2i	two inhibitors (during the cultivation of ESCs: CHIR99021 and PD184352)
Afp	alpha Fetoprotein
Alk	Anaplastic Lymphoma Kinase
ANA	6-aminonicotinamide (also 6-AN)
APC	Adenomatous Polyposis Coli
BAT-Gal	β-Catenin Activated Transcription of β-Galactosidase
Bmp	Bone Morphogenetic Protein
BRE	Bmp responsive element
C12-FDG	5-dodecanoylaminofluorescein di-β-D-galactopyranoside
CK1α	Casein Kinase 1α
DNA	desoxyribunucleic acid
DPH	diphenhydramine hydrochloride
Dvl	Dishevelled
EB	embroid body
ECM	extracellular matrix
ECVAM	European Centre for the Validation of Alternative Methods
EGFP	Enhanced Green Fluorecent Protein
EMT	epithelial to mesenchymal transition
EPA	Environmental Protection Agency
ESC	embryonic stem cell
EST	Embryonic Stem Cell Test
FACS	fluorescence activated cell sorting
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
Gsk3β	Glycogen Synthetase Kinase 3β
HDAC	Histone Deacetylase
HDACi	Histone Deacetylase inhibitor
Hnf4α	Hepatic Nuclear Factor 4α
IC_{50}	half maximal inhibitory concentration assessed on cell viability
ICM	inner cell mass
Id	Inhibitor of Differentiation or Inhibitor of DNA binding

ID_{50}	half maximal inhibitory concentration assessed on functional differentiation
iPSC	induced pluripotent stem cell
LAP	Latency-Associated Peptide
LIF	Leukemia Inhibitory Factor
Lrp5/6	Low Density Lipoprotein Receptor Protein 5/6
LTBP	Large Tgfβ Binding Protein
MAPK	Mitogen Activated Protein Kinase
MEK	MAPK kinase
Mesp1	Mesoderm Posterior 1
MET	mesenchymal to epithelial transition
MM	Micromass Test
MoA	Mode of Action
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NOAEL	no observed adverse effect level
OECD	Organisation for Economic Co-operation and Development
ONPG	ortho-nitrophenyl-beta-galactosidase
Otx2	Orthodenticle Homologue 2
p. c.	post coitum
PCR	polymerase chain reaction
Pou5f1	POU domain, class 5, transcription factor 1
PPP	pentose phosphate pathway
RA	retinoic acid
RAR	Retinoic Acid Receptor
REACH	Registration, Evaluation, Authorisation, and Restriction of Chemicals
RXR	Retinoic X Receptor
SBE	Smad Binding Element
SH2	src homology domain 2
Shh	Sonic Hedgehoc
Tcf/Lef	T-cell Factor / Lymphoid Enhancer Factor
Tgfβ	Transforming Growth Factor beta
VPA	valproic acid

WEC	Whole-Embryo Culture Test
wg	Wingless
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

10. List of Publications

Butzke, Daniel, Barbara Grune, Josephine Kugler, Michael Oelgeschläger, Andrea Seiler, Dana Sittner, Manfred Liebsch, and Andreas Luch. 2013. "The Advent of the Golden Era of Animal Alternatives." In *Animal Models for the Study of Human Disease*, 49–73. Elsevier. doi:10.1016/B978-0-12-415894-8.00003-8.

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Kugler, Josephine, Andreas Luch, and Michael Oelgeschläger. 2016. "Transgenic Mouse Models Transferred into the Test Tube: New Perspectives for Developmental Toxicity Testing In Vitro?" *Trends in Pharmacological Sciences* in press (July). Elsevier Ltd: 1–9. doi:10.1016/j.tips.2016.06.009.

11. Acknowledgment - Danksagung

Zunächst will ich mich bei Prof. Dr. Dr. Andreas Luch und dem Bundesinstitut für Risikobewertung für die Möglichkeit dieser Promotion bedanken. Besonders Prof. Dr. Dr. Andreas Luch danke ich für das Vertrauen mir dieses interessante und komplexe Thema zu überlassen und für die Bereitstellung des Arbeitsplatzes. Ich danke für die Möglichkeit mein Thema frei bearbeiten und in regen Diskussionen voranbringen zu können.

Mein besonderer Dank gilt meinem Betreuer PD Dr. Michael Oelgeschläger ohne den diese Arbeit nicht möglich gewesen wäre. Zunächst will ich mich für die Idee und Konzeption meines Themas bedanken, im Besonderen für die Bereitstellung der BRE und BAT-Gal Zellen. Noch wichtiger ist mir der Dank für das gemeinsame Entwickeln des konzeptionellen und experimentellen Vorgehens, die vielen Diskussionen, in denen ich so viele Einblicke in die Entwicklungsbiologie erhalten habe, und die Gespräche, die mich immer wieder aufgebaut und motiviert haben.

Ich danke auch Prof. Dr. Rolf Kemler für das Herstellen und Bereitstellen der beiden Zelllinien.

Ich danke ebenfalls der FAZIT-Stiftung, die es mir finanziell ermöglichte meine Doktorarbeit abzuschließen.

Einen besonderen Dank möchte ich Julian Tharmann aussprechen, der mir nicht nur die ersten Schritte in der Zellkultur näher gebracht hat, sondern auch immer wieder für mich eingesprungen ist und mir viel Arbeit im Labor abgenommen hat. In diesem Rahmen will ich allen anderen aus dem Labor danken, durch die die Arbeit und auch die Kaffeepausen viel Spaß gemacht haben.

Besonderer Dank gilt Dr. Patrick Tarnow, Dr. Iris Müller und Dr. Bettina Huhse, auf deren Hilfe ich in labortechnischen und experimentellen Fragen immer bauen konnte. Aber auch meinen Mitdoktoranden Dana, Sarah, Franzi, Franz und Marc danke ich für die vielen aufbauenden Gespräche und die gute Zeit im und außerhalb des Labors, für die vielen lustigen Stunden und das gemeinsame Durchstehen des manchmal schwierigen Weges.

Meine Familie verdient viel Dank, weil sie an mich geglaubt hat, auch wenn ich nicht immer richtig erklären konnte was ich mit den „Mikroben“ eigentlich mache. Und deshalb danke ich besonders meiner Mami, Ernie und meinem Papa, die mich in Allem unterstützt haben, finanziell und moralisch. Ohne sie wäre es mir nie möglich gewesen, diesen Weg einzuschlagen und bis hierher zu verfolgen.

Nicht zu vergessen sind meine Freunde und die fleißigen Mitsänger aus meinem Chor, die mich immer wieder daran erinnert haben, dass es ein Leben außerhalb des Labors gibt und es dadurch geschafft haben mir neue Perspektiven und Motivation zu geben.

Danke!