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ROLE OF THE HERG-CHANNEL IN ARRHYTHMIA AND TERATOGENICITY. STUDIES IN ANIMAL MODELS AND THE HUMAN EMBRYONIC HEART

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1 ABSTRACT

Background: Drugs that inhibit cardiac repolarization are associated with potentially life threatening side effects in the form of ventricular arrhythmias in humans. Animal studies show that this mechanism also is relevant for the embryo, and that the circulatory depression results in hypoxia with embryotoxicity in the form of malformations and death as a consequence. This thesis addresses the pharmacological arrhythmogenic effects on the rodent embryonic heart, and highlight the human relevance of this mechanism by characterization of the human embryonic heart.

Methods and Results: Pregnant mice were administered the hypoxia probe pimonidazole followed by phenytoin or saline at gestation day (GD) 10 or 15. Phenytoin treatment resulted in dose-dependent embryonic staining for the hypoxia probe at GD 10. At GD 15 staining was not dose-dependent and less pronounced compared to controls.

The effect on cardiac rate and rhythm of antiepileptic drugs (AEDs) was studied in cultured GD 10 mouse embryos. Phenytoin, dimethadione, carbamazepine and phenobarbital induced concentration-dependent prolongation of the inter-beat interval (IBI) and irregular arrhythmias. Exposure to combinations of AEDs in therapeutic concentrations resulted in significant increase in IBI compared to single exposure.

ECG was obtained before and after drug exposure from GD 11 rat embryos and embryonic cardiomyocytes (ECMs) cultured in multi-electrode array (MEA) culture dishes. In the embryo model phenytoin and the selective I_{Kr} blocker E4031 both induced concentration-dependent bradyarrhythmia and QT_C prolongation in cultured GD11 rat embryos. At the higher tested concentrations, phenytoin induced cardiac arrest and E4031 induced AV-nodal block. In the ECM model sensitivity to phenytoin and E4031 was similar but other arrhythmias were observed.

The distribution of Isl1+ progenitor cells and their proliferative and differentiating capacity in human first trimester embryonic hearts were determined by immunohistochemistry. $Isl1^+$ cells were present in the heart and a few were $Ki67^+$ and $troponinT^+$.

Beating clusters of human embryonic cardiomyocytes, called cardiospheres were derived from human embryonic hearts and characterized with immunohistochemistry, electron microscopy and in the MEA system. The spheres were sensitive to adrenergic stimulation with isoprenaline and displayed rate dependency of the action potential in a pacing experiment.

Expression and function of the two components of the delayed rectifier potassium current (I_K), I_{Kr} and I_{Ks}, were characterized in cardiac tissue and ECMs from human, rat and rabbit embryonic hearts. Patch clamp and quantitative RT-PCR were used. I_{Kr} was expressed and functional in all species. I_{Ks} expression was found in human and rat but not in rabbit hearts.

Conclusions: Phenytoin induces dose-dependent embryonic hypoxia. The studied AEDs induce concentration-dependent embryonic bradycardia and arrhythmia. For selective I_{Kr} blockers and phenytoin, the effects are associated with QT prolongation. This indicates that QT prolongation can be used as a biomarker for embryonic arrhythmogenicity. Rat ECMs display a similar sensitivity as the embryonic heart but respond differently to drug exposure. $Isl1^+$ cells are present in the human embryonic heart and cardiospheres derived from embryonic hearts display rate dependency of the action potential duration and sensitivity to β-adrenergic stimulation. The I_{Kr} current is expressed and functional in the human embryonic heart and in species used in teratology testing. The results support that drug-induced embryonic arrhythmia is a cause of embryotoxicity and indicate human relevance of this mechanism.

2 LIST OF ORIGINAL ARTICLES

I. Danielsson BR, Johansson A, Danielsson C, Azarbayjani F, Blomgren B, Sköld AC. (2005). *Phenytoin teratogenicity: hypoxia marker and effects on embryonic heart rhythm suggest an hERG-related mechanism.* Birth Defects Res A Clin Mol Teratol **73,** 146-53.

II. Danielsson C, Azarbayjani F, Sköld AC, Sjögren N, Danielsson BR. (2007). *Polytherapy with hERG-blocking antiepileptic drugs: increased risk for embryonic cardiac arrhythmia and teratogenicity.* Birth Defects Res A Clin Mol Teratol **79**, 595-603.

III. Genead R, Danielsson C, Wärdell E, Kjaeldsgaard A, Westgren A, Sundström E, Franco-Cereceda A, Sylvén C, Grinnemo K-H. (2009). *Early first trimester human embryonic cardiac Islet-1 progenitor cells and cardiomyocytes. Immunohistochemical and electrophysiological characterization*. Stem Cell Research **4**, 69-76.

IV. Danielsson C, Genead R, Andersson A, Sköld AC, Elsheikh E, Grinnemo KH, Hellmold H, Dencker L, Sylvén C. *Drug-induced embryonic arrhythmia – comparative in vitro electrophysiological studies in rat embryos and embryonic cardiomyocytes.* Manuscript

V. Danielsson C, Brask J, Andersson U, Stockling K, Klevenfeldt M, Wardell E, Genead R, Sköld AC, Grinnemo KH, Kjaeldsgaard A, Sundström E, Elinder F, Dencker L, Sylvén C. *Electrophysiological development in the human, rat and rabbit embryo heart – implications for drug-induced-arrhythmia related-teratogenicity.* Manuscript

3 LIST OF ABBREVIATIONS

hERG	human Ether-à-go-go Related Gene
I_{Kr}	Rapid component of the delayed rectifier potassium current
I_{Ks}	Slow component of the delayed rectifier potassium current
GD	Gestation day
ROS	Reactive oxygen species
PBN	α-phenyl-N-t-butylnitrone
AED	Antiepileptic drug
EADs	Early afterdepolarizations
IBI	Inter-beat interval
APD	Action potential duration
ECG	Electrocardiogram
QT	Interval between Q and T wave on the ECG
LQTS	Long QT syndrome
ECM	Embryonic cardiomyocyte
WEC	Whole embryo culture
FP	Field potential
FPdur	Field potential duration
Na^+	Sodium ion
Ca^+	Calcium ion
K^+	Potassium ion
Isl1	LIM homeodomain transcription factor

4 INTRODUCTION

4.1 GENERAL INTRODUCTION

Class III antiarrhythmic drugs inhibit cardiac repolarization and are associated with potentially life threatening side effects in the form of ventricular arrhythmias. Animal studies show that this mechanism also is relevant for the embryo, and that the circulatory depression results in hypoxia with embryotoxicity in the form of malformations and death as consequences. The proposed teratogenic mechanisms is illustrated in figure 1.

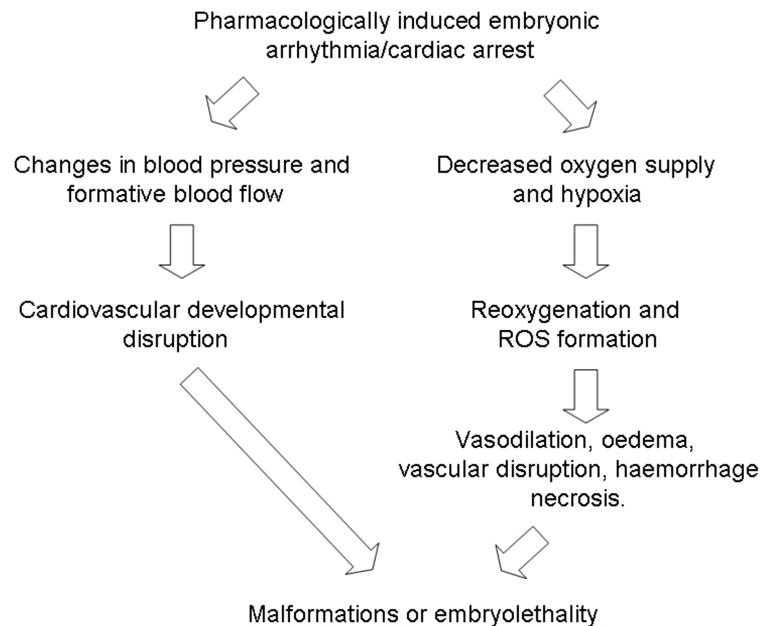

Figure 1. Schematic illustration of arrhythmia/hypoxia mechanisms of embryotoxicity**.**

There is considerable evidence that this mechanism is relevant also for drugs that inhibit cardiac repolarization as a side effect. The drug most studied in this respect is the antiepileptic drug phenytoin. Phenytoin is a known human and animal teratogen that also has the potential to induce embryonic arrhythmia.
In this thesis a thorough basis for this teratogenic mechanism is provided. I then present our studies where we investigate the effects of phenytoin, alone and in combination with other antiepileptic drugs (AEDs), on the rate, rhythm and electrophysiology of the rodent embryonic heart and rodent embryonic cardiomyocytes. The effects of the selective I_{Kr} blocker E4031 are also presented and compared to those of phenytoin. Phenytoin is in this thesis chosen as a model substance for drugs that may be teratogenic by arrhythmogenic potential on the embryonic heart as a side effect, but have other primary pharmacological effects. It is suitable since the teratogenic mechanism has been thoroughly investigated but is still under debate, and exposure data from human and animal studies is extensive. In this thesis I also present studies where the human relevance of this teratogenic mechanism is highlighted by investigations on the human embryonic heart and human cardiomyocytes.

4.2 TERATOGENICITY OF HYPOXIA

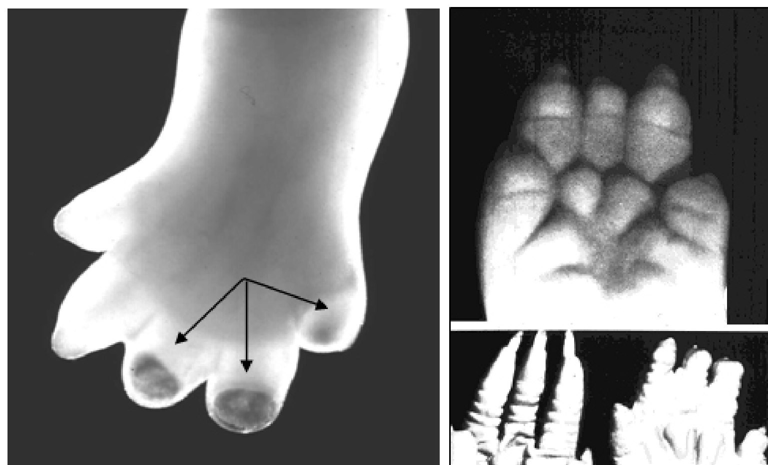

Figure 2. Distal digital reductions induced by transient intrauterine hypoxia following uterine vessel clamping. Oedema, haemorrhage and necrosis precede the malformations (left). Adopted with permission from (Brent & Franklin, 1960; Leist & Grauwiler, 1974).

Hypoxia followed by reoxygenation was one of the first teratogenic stimuli to be identified and studied, and numerous experimental studies in mice, rats and rabbits have been conducted (for review see (Grabowski, 1970). In experimental settings temporary embryonic hypoxia can be induced by reducing the oxygen tension in the air for a period, or by mechanical clamping of the uterine blood vessels, leading to impaired uteroplacental blood flow for a period (Curley & Ingalls, 1957; Brent & Franklin, 1960; Franklin & Brent, 1964). These studies show that the rat is resistant to up to 2 hours of temporary hypoxia, induced by uterine vessel clamping, during early embryonic development when the embryo is dependent on anaerobic metabolism. Hypoxia during this period results in a low incidence of congenital malformations including CNS defects such as dilated ventricles (hydrocephalus), orofacial clefts, and heart defects (Franklin & Brent, 1964; Leist, 1973).

When the embryonic heart starts beating (GD 9 in rat) the metabolism shifts to aerobic and the sensitivity to hypoxia induced malformations increase. Between GD 12 and 16 the embryos are the most sensitive, and are in general unable to survive 60 minutes of hypoxia. Shorter periods of hypoxia of 30-45 minutes during this period of development induce a range of malformations, that vary depending on day of gestation of the hypoxic event. For example, high incidence of distal digital defects (brachy/oligodachtyly) on the fore paws on GD 13 and hind paws on GD 14 (see figure 2). Other defects include cardiac, tail, face, and urogenital abnormalities (Leist & Grauwiler, 1974; Webster *et al.*, 1987), as well as skeletal defects. The skeletal defects observed show a progressive cranial to caudal shift depending on during what developmental stage the hypoxic event occurs (Ingalls & Curley, 1957; Degenhardt, 1958; Murakami, 1963; Morawa & Han, 1968; Leist, 1973).

The exact mechanism how temporary hypoxia gives rises to tissue damage is not fully understood. During embryonic development, hypoxia may induce cellular growth retardation, changes in differentiation capacity or death (Naujoks, 1953; Bronsky *et al.*, 1986). This could in turn result in embryonic death or growth retardation that would be more pronounced in the areas with most cell proliferation.

Another complementary mechanism of hypoxia-induced embryonic tissue damage is ROS formation. There is considerable evidence that, just like in the adult, ROS formation during reperfusion (reoxygenation) plays an important role.

In the adult, it is well established that temporary hypoxia result in ROS generation in the vascular epithelium, increased vascular permeability, oedema and cellular injury/death (Li & Jackson, 2002). The same type of early changes precede tissue damage and specific malformations, such as cleft lip/palate and digital reduction defects, in the embryo after uterine vessel clamping (Leist & Grauwiler, 1974; Webster *et al.*, 1987) (see figure 2). Further, ROS has been identified in embryonic tissues after periods of hypoxia followed by normoxia (Fantel *et al.*, 1995). ROS has also been specifically associated with cardiac developmental disturbances as studies show that the developing embryonic heart, and especially the out flow tract, is sensitive to ROS-induced cellular damage (Fisher, 2007).

4.3 ADULT AND EMBRYONIC CARDIAC ELECTROPHYSIOLOGY

4.3.1 Effects of selective hERG blocking drugs

Delayed inward rectifier currents and pharmacological effects of class III antiarrhythmic drugs

Adult heart. The delayed rectifier potassium current (I_K) is the major repolarizing outward K^+ current in cardiac myocytes during phase III of the action potential in adult humans. This current is composed of two components, I_{Kr} (rapidly activating), and I_{Ks} (slowly activating), of which I_{Kr} (expressed by the product of the KCNH2/hERG gene) appears to be the most important repolarizing current in the heart. Class III antiarrhythmic drugs, of which many are selective I_{Kr} blockers, act on repolarizing cardiac ion channels and prolong the action potential duration (APD). On the surface ECG, the APD prolongation is seen as a prolongation of the QT interval (see figure 3) or the QT_C interval (QT corrected for heart rate). The antiarrhythmic effect is achieved through prolongation of the effective refractive period. In rare cases, class III antiarrhythmic therapy results in a polymorphic type of ventricular tachycardia called Torsades de Pointes, which is preceded by severe QT prolongation and early afterdepolarizations. Because of this, class III antiarrhythmics are used under strict safety surveillance.

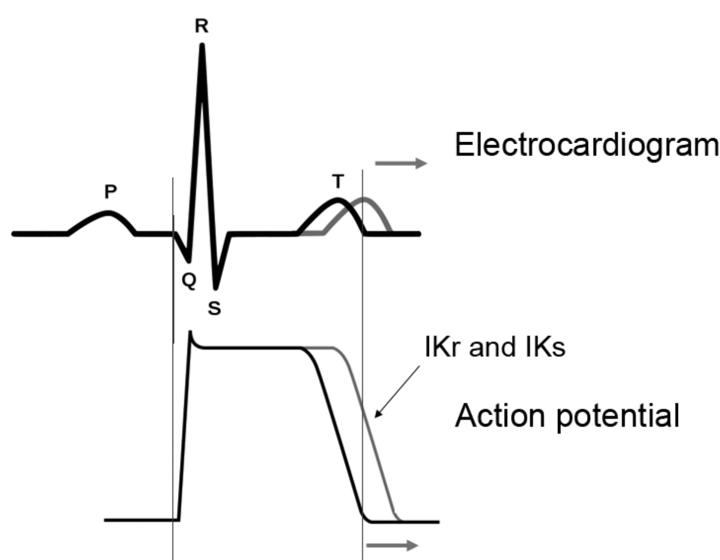

Figure 3. Schematic illustration of the relation between the electrocardiogram and the ventricular action potential. Pharmacological inhibition of IKr and IKs in phase 3 of the action potential prolongs the APD and the QT interval.

The proarrhythmic effects of selective I_{Kr} blocking class III antiarrhythmic drugs such as almokalant, dofetilide and ibutilide can be reproduced in animal models. The rabbit heart appears to be especially sensitive to I_{Kr}-inhibition induced arrhythmia, and is used to test this property in the development of new drugs (Carlsson *et al.*, 1993). However, the expression of the two I_K components differs between mammalian species. For instance, both I_{Kr} and I_{Ks} are present in guinea pig (Brouillette *et al.*, 2007), and human ventricular myocytes (Li *et al.*, 1996), whereas only I_{Kr} appears to be important in the rabbit heart (Cheng & Incardona, 2009). In contrast, the pure I_{Kr} blockers almokalant and dofetilide have no effect on the action potential duration in the adult rat or mouse (Abrahamsson *et al.*, 1994; Wang *et al.*, 1996), why the these species are considered as less relevant for identifying arrhythmogenic potential of I_{Kr} blocking drugs. Instead, the dominant repolarizing outward current in the adult rat is I_{to} and it appears that the action potential is too short to allow activation of I_K to any considerable extent (Tande *et al.*, 1990).

Embryonic heart. The species differences in delayed rectifier expression and function observed in the adult appear not to be the same during embryonic development. Studies with selective I_{Kr} blockers such as E4031 and dofetilide, show that functional I_{Kr} is important for embryonic cardiac repolarization in several species including mouse (Davies *et al.*, 1996; Wang *et al.*, 1996), chicken (Krogh-Madsen *et al.*, 2005), rat (Abrahamsson *et al.*, 1994) and zebrafish (Langheinrich *et al.*, 2003). The importance of I_K for cardiac repolarization in the human embryo has not been studied but I_{Kr} is expressed and essential for repolarization in cardiomyocytes derived from human embryonic stem cells (He *et al.*, 2003).
Transmembrane recordings from rat embryonic hearts (GD 13) showed that exposure to increasing concentrations of the selective I_{Kr} blocker almokalant induced concentration-dependent bradycardia and arrhythmia in the rat embryo. The bradycardia was accompanied by APD prolongation and after extensive prolongation of the action potential, almokalant induced spontaneous deflection of the membrane potential consistent with EADs, which preceded the bradycardic arrhythmia.
Another, more widely used, method to study pharmacological effects on the embryonic heart is visual analysis of cardiac activity in cultured embryos. Culturing rodent embryos on different days of gestation show that the embryonic heart reacts in a concentration-dependent manner with bradycardia, irregular rhythm and cardiac arrest in the presence of the class III antiarrhythmic drugs almokalant, dofetitilide and L 691.121 (Ban *et al.*, 1994; Spence *et al.*, 1994; Webster *et al.*, 1996). These effects are reversed after wash out. The sensitivity of the embryo to react with arrhythmia is however dependent on gestational age, and selective I_{Kr} blockers do not induce rhythm abnormalities in the rat embryo after GD 14 (Webster *et al.*, 1996). During prenatal and postnatal development the rat cardiac action potential undergoes dramatic developmental changes (Couch *et al.*, 1969) and the heart rate increases. The embryonic and early fetal action potential has a pronounced plateau phase and frequency-dependent duration. After birth however, the ventricular AP is spike like and has lost the rate-dependent properties. The embryonic heart rate is 120-160 beats per minute while it is up to 1000 beats per minute in the adult. The developmental processes behind the changes in sensitivity to I_{Kr} blocking drugs and action potential properties are not fully understood, but are

likely to be a combination of ion channel regulation and expression, morphological changes and development of cardiac innervation.

More recently, another method has been used for investigating pharmacological effects in spontaneously beating embryonic cardiomyocytes from mouse, rat and chicken, cardiac tissue samples as well as isolated embryonic mouse hearts (Halbach *et al.*, 2003; Meyer *et al.*, 2004; Reppel *et al.*, 2004; Yeung *et al.*, 2009). Micro electrode arrays are used to record extracellular electrophysiological signals and the method has been proposed as a screening tool for QT related pharmacological cardiac toxicity in the adult (Meyer *et al.*, 2007). Concentration-dependent prolongation of measured field potentials could be induced by antiarrhythmic drugs and selective I_{Kr} blocker E4031 in embryonic chicken cardiomyocytes (Meyer *et al.*, 2004).

Teratogenicity of selective I_{Kr} blocking drugs

There is no data available on teratogenic effects of class III antiarrhythmic drugs in human pregnancy outcome studies. In conventionally designed teratology studies with class III antiarrhythmic drugs almokalant, dofetilide, ibutulide, and L 691.121 (Abrahamsson *et al.*, 1994; Ban *et al.*, 1994; Spence *et al.*, 1994; Marks & Terry, 1996), the pharmacologically induced embryonic bradycardia, tachyarrhythmia and cardiac arrest have been proposed to explain the observed high incidences of embryo/fetal death and decreased fetal weights and observed malformations in the few surviving embryos. However, using single dosing regimens on specific gestation days it is possible to induce high incidences of stage specific malformations without high incidences of embryonic death. Single dose studies have also shown that the class III antiarrhythmic drugs almokalant and dofetilide, induce identical stage specific external and visceral malformations (orofacial clefts, ventricular septum defects, tail defects or distal digital defects) on a specific day of gestation, as interrupted oxygen supply following uterine vessel clamping on the same day of gestation. (Webster *et al.*, 1996; Wellfelt *et al.*, 1999; Skold *et al.*, 2001) (see figure 4). Similar malformations have been induced by drugs that have potent I_{Kr} blocking potential as a side effect, cisapride (Skold *et al.*, 2002) and astemizole (Nilsson *et. al.*, in press).

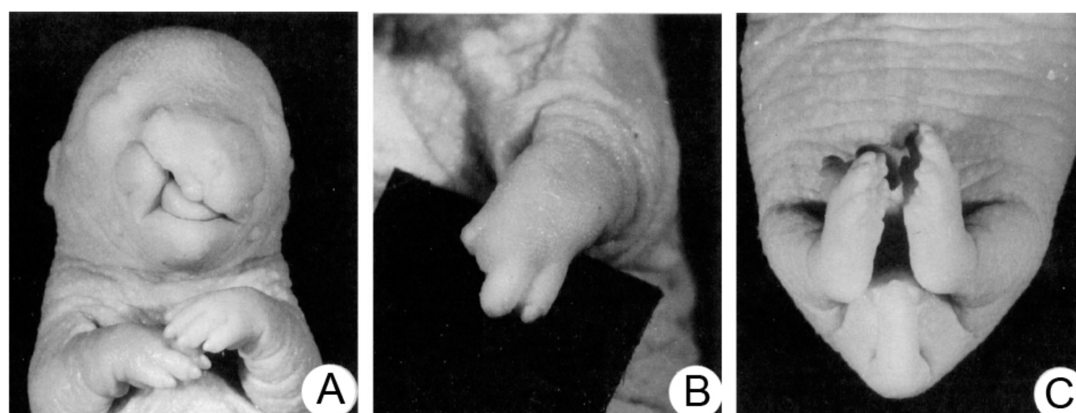

Figure 4. Malformations in rat fetuses induced by dosing of almokalant on a single day of gestation. Dosing on GD 11 induced cleft lip and palate (A) and tail reductions (C). On GD 13 dosing resulted in distal fore limb reductions (B). Adopted with permission from (Webster *et al.*, 1996).

Further support for the idea that the malformations are the result of arrhythmia with subsequent hypoxia, is the finding that the embryotoxicity and malformations can be induced during the same period when the embryonic heart is sensitive to I_{Kr} block induced arrhythmias (GD 9-14), but not prior to or after this period. In addition, the same type of pathological findings (oedema, vascular disruption, haemorrhage and necrosis) precede stage specific orofacial clefts and distal digital defects induced by I_{Kr} blockers or hypoxia following uterine vessel clamping (Webster *et al.*, 1996).
This form of tissue damage is however not found to precede the cardiovascular anomalies. Arrhythmia-induced disruption of normal blood pressure and formative blood flow may instead play an important role in the observed ventricular septal and other cardiac defects. Studies have shown that mechanical interference with venous blood flow to the heart or with the left chamber (Hogers *et al.*, 1997; Tobita & Keller, 2000), as well as pharmacologically altered rhythm (Gilani & Silvestri, 1977), during cardiac development result in cardiac malformations.

Evidence of embryonic hypoxia and ROS generation

Further efforts have been made to clarify the relation between arrhythmia and malformations. A study where the hypoxia marker pimonidazole was used shows that a teratogenic dose of almokalant, administered in vivo to pregnant rats during the period when the embryo heart is sensitive to I_{Kr} block, induces embryonic hypoxia and irregular bradycardia. In, contrast, no difference in heart rate or hypoxia compared to control was observed at a later gestational day after the sensitive period (Danielsson *et al.*, 2003b) (see figure 5).
The ROS scavenger PBN, with the ability to capture and neutralize ROS, has been used to further investigate the role of ROS in almokalant teratogenicity. Pre-treatment with PBN resulted in a significant decrease in the incidence of almokalant-induced defects both on GD 11 and 13. No fetuses with orofacial clefts, ventricular septum defects, tail defects or distal digital defects were observed in almokalant-treated dams after pre-treatment with PBN (Wellfelt *et al.*, 1999).

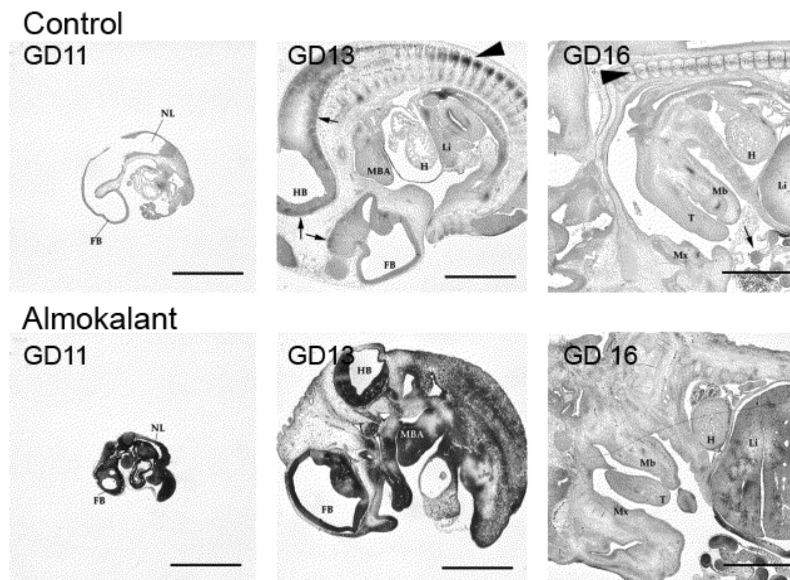

Figure 5. Increased staining for the hypoxia probe pimonidazole induced by the selective I_{Kr} blocker almokalant on gestation day 11 and 13. No significant increase in staining could be induced on GD 16 which is after the period when the embryonic heart is dependent on I_{Kr} for repolarization. Adopted with permission from (Danielsson *et al.*, 2003b).

4.4 ANTIEPILEPTIC DRUGS BLOCKING CARDIAC ION CHANNELS AS A SIDE-EFFECT

4.4.1 Phenytoin

Pharmacological action and effects on the adult and embryonic heart

The antiepileptic drug phenytoin is pharmacologically active by stabilizing membranes of neurons and is used in treatment of grand mal seizures. The primary pharmacological effect of phenytoin is inhibition of voltage gated sodium (Na^+) currents, but it also acts on voltage gated calcium (Ca^{2+}) and potassium (K^+) ion channels involved in action potential propagation or burst generation (Rogawski & Porter, 1990; Molnar & Erdo, 1995). Phenytoin also blocks cardiac sodium and calcium ion channels (Yatani *et al.*, 1986; Barber *et al.*, 1991) and has relatively recently been shown to inhibit I_{Kr} at clinically relevant concentrations (Danielsson *et al.*, 2003a)

Phenytoin is associated with cardiac arrhythmia in humans. At least 15 patients have developed severe bradycardia, including eight cases of phenytoin-induced arrhythmia evolving to asystole and death (Tomson & Kennebäck, 1997). Phenytoin also induces bradycardia/arrhythmia in healthy subjects. Marked sinus bradycardia leading to syncope was observed in four of 15 young volunteers given phenytoin by intravenous injection (Barron, 1976). Phenytoin is however not associated with QT prolongation or Torsades de Pointes arrhythmias, despite I_{Kr} blocking properties at concentrations considered outside proposed clinical safety margins (Redfern *et al.*, 2003).

In rodent studies phenytoin induces embryonic bradycardia and ventricular arrhythmias/cardiac arrest in both cultured rat and mouse embryos during the same period as arrhythmias can be induced by selective I_{Kr} blockers (Abrahamsson *et al.*, 1994; Azarbayjani & Danielsson, 2001). The observed effects were reversible after wash out and started at clinically relevant concentrations.

The analysis of drug-induced embryonic cardiac effects has been mainly limited to visual observation with the exeption of the study by Abrahamsson (Abrahamsson *et al.*, 1994), and little is known about the normal embryonic cardiac electrophysiology and mechanisms underlying both class III antiarrhythmic and phenytoin-induced arrhythmia. In this thesis I have developed and refined techniques to improve embryonic arrhythmia-analysis. In paper I and II we utilize a new digitalization tool for more objective analysis of cardiac activity. In paper IV we combine the whole embryo culture and the multi-electrode array method to study the electrophysiological character of the embryonic cardiac effects of phenytoin and the potent selective I_{Kr} blocking drug E4031, and investigate if the arrhythmia is associated with QT prolongation.

MEA recordings from primary embryonic cardiomyocytes has been proposed as a QT screening tool for the adult heart (Halbach *et al.*, 2003; Meyer *et al.*, 2004). In the second part of paper V, we culture primary embryonic cardiomyocytes in the multi-electrode array system and study the potential of this model to predict embryonic arrhythmia.

Teratogenicity of phenytoin

Antiepileptic drug use during human pregnancy is one of the most common potentially teratogenic exposures, occurring in 0.4–0.6% of all pregnancies (Holmes *et al.*, 2001). Despite this, women with epilepsy require continuous treatment with antiepileptic drugs since the risks for the mother and the unborn are considered more severe than the two- to threefold increased risk for malformations.

Phenytoin may be the antiepileptic drug where most clinical pregnancy outcome data is available. It is an established human teratogen and has been reported to cause fetal adverse effects in mice, rats and rabbits. The increased risk of malformations has been

shown to be unrelated to the severity of the mother's epilepsy (Holmes *et al.*, 2001). The pattern of fetal adverse effects, described as "Fetal Hydantoin Syndrome" in humans (Hanson & Smith, 1975), is very similar across species (Finnell & Dansky, 1991). It consists of embryonic death, growth retardation as well as a pattern of structural abnormalities mainly characterized by orofacial clefts, distal digital reductions and cardiovascular defects. All of the reported major fetal adverse structural defects included in the fetal hydantoin syndrome in human studies, have been possible recreate in animal studies.

Accumulating evidence support the idea that the embryotoxicity and teratogenicity of phenytoin is caused by the same mechanism as that of selective I_{Kr} blocking drugs. The malformations induced by phenytoin are very similar to those induced by selective class III antiarrhythmic drugs and hypoxia, and are preceded by haemorrhage and necrosis (orofacial clefts and digital reductions) (Mercier-Parot & Tuchmann-Duplessis, 1974; Brown *et al.*, 1985; Azarbayjani & Danielsson, 2001). Further, phenytoin inhibits cardiac ion channels including I_{Kr} and induces concentration-dependent embryonic arrhythmias and cardiac arrest. A study was designed to investigate this idea more in detail. The results showed that a dosing regimen of phenytoin not causing any fetal adverse effects did not cause any arrhythmogenic effects on the embryonic heart. In contrast, dosing regimens causing slight and marked bradyarrhythmia, resulted in slight and marked developmental toxicity, respectively (Danielsson *et al.*, 2000).

There are also several studies that support the involvement of ROS in phenytoin teratogenicity. In rodent studies, phenytoin-induced malformations can be reduced with pretreatment with the ROS scavenger PBN as well as antioxidants including vitamin E (Winn & Wells, 1995; Yu & Wells, 1995).

In order to investigate if the phenytoin-induced arrhythmia, ROS generation and malformations also are associated with embryonic hypoxia (as is the case for selective I_{Kr} blocking drugs), we use the hypoxia probe pimonidazole in paper I.

4.4.2 Dimethadione, phenobarbital and carbamazepine

Like phenytoin, the antiepileptic drugs phenobarbital, trimethadione (via its active metabolite dimethadione), and carbamazepine have been associated with human teratogenicity (Dansky & Finnell, 1991). The observed pattern of malformations is similar to that of phenytoin and class III antiarrhythmic drugs, including growth retardation, defects related to growth retardation (e.g., microcephaly), and structural malformations, such as heart defects, orofacial clefts, and limb defects. In rodent teratology studies, the malformations can be recreated with dimethadione. Cleft palate in the term fetus was preceded by haemorrhage in the nasopharyngeal part of the palate in embryonic tissues 24 and 48 hours after maternal administration of dimethadione in the same way as reported for phenytoin. Further, pretreatment with PBN severely reduced the teratogenic potential of dimethadione, providing evidence for ROS mediated damage to the developing tissues (Azarbayjani & Danielsson, 2002).

Dimethadione, phenobarbital and carbamazepine have also been shown to inhibit I_{Kr} in a concentration-dependent manner (Danielsson *et al.*, 2003a) and dimethadione induces concentration-dependent bradycardia and arrhythmias in embryo culture studies (Azarbayjani & Danielsson, 1998).

In humans, the risk for birth defects, especially for multiple major malformations, is greatly increased after treatment with two or more of these antiepileptic drugs (poly-

therapy) (Dansky & Finnell, 1991; Pennell, 2003). In paper II we investigate the effects on embryonic cardiac rate and rhythm after exposure to increasing concentrations of these antiepileptic drugs. We also mimic the increased risk of polytherapy by exposing the embryos to combinations of clinically relevant concentrations of anti epileptic drugs, in order to study potential additive effects on the embryonic heart.

4.5 DEVELOPMENT OF THE EMBRYONIC HEART

4.5.1 Isl1$^+$ cardiac progenitor cells and cardiospheres

During organogenesis different cell populations migrate and give rise to distinct parts of the heart. The studies, on which the knowledge on cellular origins in cardiac development is based, are mainly limited to investigations in mouse and rat. The results show that the heart is derived from mesoderm that responds to signals from the surrounding tissue to develop into two different heart fields (Kelly & Buckingham, 2002; Buckingham *et al.*, 2005). The earliest population of cardiac progenitors, referred to as the first heart field, gives rise to the linear heart tube and subsequently parts of the atria and the left ventricle. Cells of the second heart field are derived from the pharyngeal mesoderm that lies anterior and dorsal to the heart tube and subsequently gives rise to the outflow tract, right ventricle, part of the atria, and a few portions of the left ventricle. The LIM homeodomain transcription factor Islet-1 (Isl1) marks a cell population that constitutes the second heart field (Cai *et al.*, 2003) and should thus represent a true endogenous cardiac progenitor cell. In vivo cell lineage tracing in mouse embryos using the Cre-loxP strategy has confirmed that Isl1$^+$progenitors contribute to more than two-thirds of the cells in the embryonic heart including parts of the conduction system, and endothelial/ smooth muscle cells throughout the proximal aorta, pulmonary trunk, and the stems of the proximal left and right coronary arteries (Cai *et al.*, 2003; Moretti *et al.*, 2006; Sun *et al.*, 2007). The Cre-loxP strategy describes Isl1 promoter expression but not directly the protein expression of Isl1 and not to what extent Isl1 cells proliferate and differentiate.

The search for progenitor cell types with the inherent capability to differentiate into the cell types that make up the heart aims to increase the understanding of cardiac development, as well as to identify a possible candidate cell for cardiac regeneration therapy. Further, in vitro expansion of progenitor cell progeny may provide a source of human embryonic cardiomyocytes.

In paper III we describe the distribution of Isl1$^+$progenitors and their proliferative and differentiating capacity in the early first trimester human embryonic heart. Further, we derived proliferating, spontaneously beating clusters of cardiomyocytes, called cardiospheres, in culture. The cardiospheres were characterized in the multi-electrode array system regarding electrophysiological properties, susceptibility to adrenergic stimuli and rate dependence of the field potential.

4.5.2 I_K channel expression and function in cardiac development

As described earlier, the major repolarizing force in the human adult heart is the delayed rectifier potassium current IK, with the two components I_{Ks} and I_{Kr}. The IK current is conducted by voltage-gated potassium channels of the K_V family. Voltage gated potassium channels consist of tetramers of pore forming α-subunits that co-assemble with regulatory β-subunits that can alter conductance, gating snd pharmacological prop-

erties of the channels. The gene KCNH2 (also known as hERG) encodes for the pore-forming α-subunit $K_v11.1$ that forms the channel generating I_{Kr}. In vitro the β-subunit MiRP1 (encoded for by the KCNE2 gene) regulates $K_v11.1$ channel function (Abbott *et al.*, 1999). The gene KCNQ1 codes for the pore-forming α-subunit that together with the β-subunit MinK (encoded for by the KCNE1 gene) forms the channel generating I_{Ks} (Sanguinetti *et al.*, 1996). Two other β-subunits, MiRP2 and MiRP3 (encoded for by KCNE3 and KCNE4, respectively) regulate Kv7.1 channel function in vitro but their relevance in vivo has yet to be fully elucidated (Abbott *et al.*, 2007).

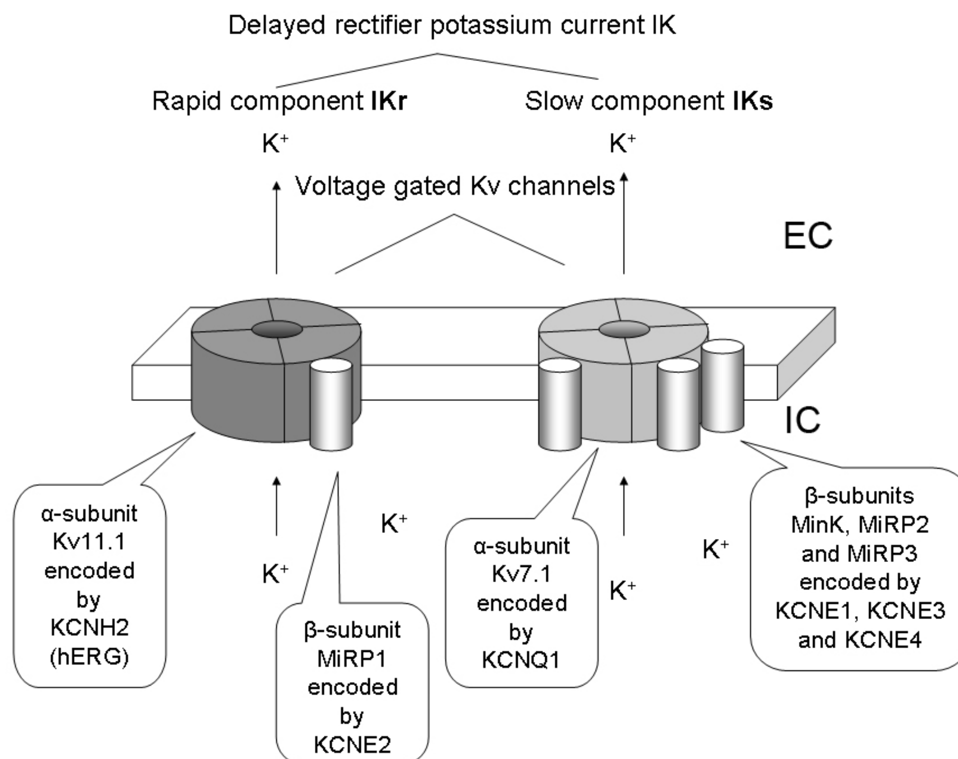

Figure 6. Schematic illustration of the two components of the delayed rectifier potassium current I_K, with names of ion channel subunits and their respective encoding genes indicated. IC = intracellular, EC = extracellular.

Due to the risk of proarrhythmia, there is extensive literature on I_{Kr} and cardiac repolarization in the adult heart across species, and large species differences in the importance of the I_K currents are apparent. On the contrary, very little is known about the electrophysiological development of the embryonic heart. The available data again depicts the rodent situation, and to our knowledge, the I_K current has nor been studied in the human embryonic heart.

In the mouse, cardiac whole mount mRNA in situ hybridization shows that KCNH2 and KCNQ1 are expressed and evenly distributed from GD 9.5 and throughout cardiac development. KCNE1 is initially evenly expressed but is confined to the ventricular myocardium in later stages of development. KCNE2 expression is restricted to the atria, while KCNE3 is initially expressed throughout the myocardium but is later confined to the atria. Protein expression of KCNH2 and KCNQ1 correlated with the mRNA expression (Franco *et al.*, 2001). Protein expression of KCNE3 was found in both the atria and ventricle (de Castro *et al.*, 2006).

Patch clamp studies in mouse embryonic and fetal cardiomyocytes (GD 11-13 and 17-20 respectively) show that I_{Kr} is present in equal amounts in atrial and ventricular tissue and that expression levels are constant between early and late cardiac development. I_{Ks} was not found in early or late stage atrial cells, but is present in ventricular cells at both stages (Davies *et al.*, 1996).

In the rat, in situ hybridization shows that expression of KCNE2 is confined to the myocardium in late cardiac development (GD 14.5, 16.5 and 18.5) and that it co expresses spatially and temporally with KCNH2 which was homogeneously distributed in the myocardium (Chun *et al.*, 2004). The importance of the species difference in KCNE2 distribution is not known, but indicates that there are species differences in I_K function also during embryonic life.

In electrophysiological studies in fetal rat atrial and ventricular tissue, class III antiarrhythmic drugs provoke a dose-dependent prolongation of the action potential duration, and a prolongation of inter beat interval. With increasing dose, arrhythmia and/or early after depolarisations are induced. A current sensitive for almokalant and resembling I_K is found in fetal and adult cultured cardiomyocytes (Abrahamsson *et al.*, 1994).

Regulatory guidelines require animal studies in two species, one rodent and one non-rodent (usually rat and rabbit) to assess the risk for drug-induced teratogenicity in human pregnancy. Malformations as well as embryonic arrhythmias can be induced in both these animal species by selective IKr blocker and antiepileptic drugs, indicating a common arrhythmia/hypoxia related mechanism of teratogenicity. It is however very difficult to make comparisons and extrapolations across species, since the knowledge about electrophysiological development in humans as well as in species used in teratology testing is so scarce. In paper V, we attempt to illuminate the possible human relevance of the animal findings by comparing delayed-rectifier potassium channel function and expression in embryonic heart and cardiomyocytes from rabbit, rat and human at different points of cardiac development.

5 AIMS

- To investigate the potential of phenytoin to induce embryonic hypoxia and to use the new digitalization method to characterize phenytoin-induced embryonic arrhythmia.

- To investigate if the antiepileptic drugs and human teratogens phenytoin, phenobarbital, dimethadione and carbamazepine induce embryonic cardiac arrhythmia, alone or in combinations at clinically relevant concentrations.

- To describe the distribution, proliferative and differentiating capacity of $Isl1^{+}$ progenitor cells in the early first trimester human embryonic heart. To electrophysiologically characterize beating cardiospheres derived from human embryonic hearts.

- To electrophysiologically characterize the rat embryonic heart and the effects of phenytoin and E4031 on arrhythmia and QT prolongation. To study the potential of rat embryonic cardiomyocytes to predict the arrhythmogenic effects seen in cultured embryos.

- To study the expression and function of I_K ion channels in human, rat and rabbit embryonic hearts during embryonic development.

6 MATERIALS AND METHODS

6.1 ANIMALS AND ETHICS

C57BL/6J mice from B&K Universal AB (Sollentuna, Sweden) were used in paper I and II. Time-mated Sprague Dawley (SD) rats (Charles River, Germany) were used in paper IV and V. New Zeeland White rabbits (Charles River, Germany) were used in paper V. All studies involving animals or human material were approved by the ethical committee affiliated to Uppsala University, Karolinska Institutet or Karolinska University Hospital.

6.2 ANALYSIS OF EMBRYONIC CARDIAC ACTIVITY

6.2.1 Embryo culture – Paper I, II and IV

Paper I and II

Pregnant mice were killed by cervical dislocation and embryos were prepared for culture using a modified technique of New (1978). In brief, the abdomen was opened, the uterus excised, and decidual swellings removed under aseptic conditions. The decidua and Reichert's membrane were removed from each swelling and the embryos, with intact yolk sac and ectoplacental cone, were placed in sterile culture bottles containing 2.5 ml of culture medium. The culture medium consisted of immediately centrifuged rat serum and Tyrode's buffer + 50 IU/ml penicillin and 50 mg/ml streptomycin. The culture bottles were gassed with 40% O_2, 55% N_2, and 5% CO_2 (vol/vol/vol) and capped tightly and cultured in a rotating culture system at 37°C and 40 rpm. After 60 min, the heart activity was videotaped using a microscope and then analyzed after digitalization (Skold *et al.*, 2002). The embryos were kept on a heated stage to maintain the bottles at 37°C. Embryos were selected based on the following criteria: 1) morphologically normal appearance; 2) good yolk sac circulation; 3) heartbeat of at least 120 beats per min. Embryos not fulfilling these criteria were discarded. After the selection of embryos, PHT was added to each culture bottle (controls were treated with the solvent) and the bottles were again gassed for 2 min. After another 30 min after the heart rhythm was again videotaped using a microscope and then analyzed after digitalization.

Paper IV

A modified method of whole embryo culture (WEC) as described in paper I and II was used. Pregnant SD rats were sacrificed in a CO_2 chamber followed by cervical dislocation on GD 11. The uterus was removed and the embryos were dissected free from surrounding tissue and membranes with sharp forceps under a stereomicroscope at room temperature in sterile HBSS without Ca^{2+} and Mg^{2+} (GIBCO, Invitrogen, USA). In order to facilitate optimal contact between the heart and the electrodes the embryos were cultured outside the yolk sac. A small rift was made in an avascular area of the yolk sac and the amniotic membrane was opened after which the embryo was pushed through the opening. Embryos were put in MEA culture dishes (Multi Channel Systems, Reutlingen, Germany), containing high glucose DMEM (GIBCO, Invitrogen, USA), placed in sealable plastic containers, gassed for two minutes with 95 % O_2 and 5 % CO_2 and cultured in an incubator for one hour at 37°C before ECG recording. Total culture time of the embryos was two hours.

6.2.2 Video digitalization – Paper I and II

The digitalisation of the embryonic heart rhythm was performed by displaying the tapes on a monitor while a handheld probe was placed on the spot of the pulsating embryonic heart. The light sensitive probe designed for this purpose generates a signal that is fed to an analog/digital converter (Pasco Scientific® CI-6450), which is governed by analytical software (Pasco Scientific® CI-6870C). The signal is in the shape of a graph showing the difference in light intensity (depending on the amount of blood in the heart compartment) on a spot of the heart over time. The software was used to calculate IBI as the time between peaks, and introduces objective methods for measurement of IBI variability in embryonic heart rhythm. This technique was applied to investigate rhythm disturbances in all video recorded embryos.

6.2.3 Multi-electrode array embryo ECG – Paper IV

The multi-electrode array method is described in detail in publications by others (Stett *et al.*, 2003; Meyer *et al.*, 2004). Embryos were cultured in MEA culture dishes. Each MEA culture dish had 60 flat, round, substrate-integrated titanium electrodes in an 8×8 grid and an integrated reference electrode (electrode diameter 30 μm and interelectrode distance 200 μm) (see figure 7). The MEA was connected to an amplifier with a heated stage (Multi Channel Systems, Reutlingen, Germany).

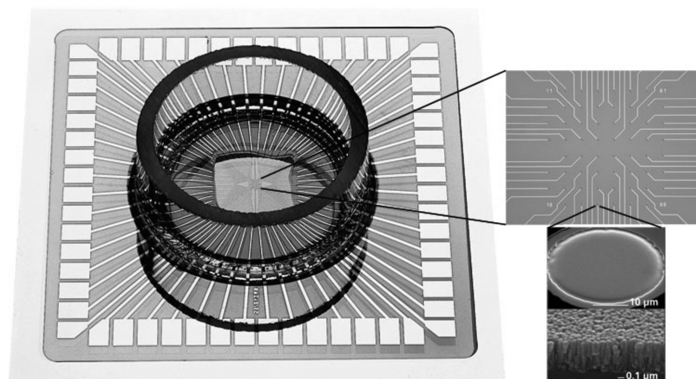

Figure 7. Multi-electrode array culture dish. Micro electrodes in the centre are enlarged (right).

ECG recordings were carried out in MEA culture dishes on a 37 °C heated stage. Prior to recordings embryos were manipulated into position over the electrodes and medium was taken away to increase surface tension on the embryos as to facilitate better contact between the heart and the electrodes. The time between the embryos were taken out of the incubator until recording started was approximately 2 minutes. Each recording started when a good signal was acquired and lasted for 45 seconds. Two ECG recordings were carried out on each embryo, the first after one hour of incubation with control medium and recording 2 one hour later after incubation with either control medium + vehicle or active substance. Only embryos meeting the predetermined inclusion criteria were randomised into control or treatment groups and allowed to go on to recording 2. The inclusion criteria were: normal morphological appearance, good yolk sac circulation, regular rhythm and IBI (time in ms between the heart beats) of ≤520 ms (corresponding to a heart rate of ≤ ~ 115 beats per minute).

6.3 DETECTION OF EMBRYONIC HYPOXIA WITH PIMONIDAZOLE – PAPER I

Hypoxia probe studies

Pregnant mice were administered the hypoxia marker pimonidazole hydrochloride (60 mg/kg) intravenously (i.v.) followed by phenytoin (100 and 150 mg/kg) on GD 10 and GD 15. Controls were administered corresponding volumes of saline on the same GD. There were 4 animals per treatment regimen (24 total pregnant mice). At 2.5 hr after the pimonidazole dose, cervical dislocation was performed before the cesarean section and the embryos were removed from the uterus. The embryos were then placed in 4% neutral buffered formaldehyde solution for subsequent immunohistochemistry.

Immunohistochemistry

A detailed description, of the method is described by Danielsson (Danielsson *et al.*, 2003b). The specimens were immersed in 4% neutral buffered formaldehyde solution.Three sagittal sections were taken from each embryo. The sectioning was started in the median plane of each embryo. Thereafter, the sections were deparaffinized and rehydrated. As pretreatment, a 0.05% Pronase solution (DAKO S 2013) in 0.05 M TRIS-buffered saline (TBS) was used for 10 min in room temperature. Subsequently, the slides were rinsed in PBS. A monoclonal primary antibody (NPI Hypoxyprobe-1Mab1; NPI, Belmont, MA) was used, in a 1:200 dilution (DAKO S 2022). The immunohistochemical staining was performed in a DAKO TechMate (Glostrup, Denmark), using the MSIP program. All other reagents and accessories (except primary antibody) used were provided for the instrument. Negative controls were run with a primary antibody intended for use as negative control (DAKO ChemMate Negative Control H 0960). This procedure resulted in negative staining.

Image analysis

The sections were analyzed with a computer- assisted image analysis system consisting of an illuminating box and a black-and-white charged-coupled device (CCD) video camera connected to a Macintosh Quadra 950 (Apple Computer, Kista, Sweden) computer equipped with a frame-grabber. A detailed description of the system setup is given in (d'Argy *et al.*, 1990). The light from the illuminating box was allowed to stabilize, and thereafter the system was calibrated with a set of Kodak Wratten (Upplands Vasby, Sweden) gray filters of known densities. This enabled the system to set exact values for black and white and from these values determine all grayscale densities inbetween. The program could distinguish 256 gray scale values, where black = 0 and white = 255. The next calibration step was the measurement of background illumination, without any specimen on the illuminating box. The background illumination was set to white. Thereafter, the specimen was placed on the illuminating box and an image of the specimen was captured and saved in 8-bit grayscale mode under the control of the image analysis software. The image size was equal in all groups. Each image consisted of 240 x 320 pixels. Three measurements were made of the area around the embryo, since the glass in the slide and coverslip as well as the mounting medium also absorb a small amount of light. These measurements were performed for the subsequent subtraction of background density. Measurements of the whole embryo and selected organs of interest were made. Finally, the background density was subtracted from the density of the region of interest,

6.4 CELL CULTURE

6.4.1 Cardiosphere culture – Paper III

Aborted human material (gestational weeks 5 to 10) was transported directly from the operating room to the dissection room where the heart was the first organ to be identified to reduce the time of ischemia. Gestational age was determined by examination of the fetus for morphological landmarks (England, 1990). Time between abortion and culture preparation was 0.5–1 h.

The cardiac material was minced into small pieces and digested in 3–5 mL of collagenase solution (Collagenase type 2, CLS-2 Worthington 160 U/mL in HBSS without Ca and Mg) during continuous stirring for 5 min at 37 °C in an incubator at humidified atmosphere containing 5% CO_2. The first fraction of cells was discarded while the remaining tissue fragments in the supernatant were repeatedly digested in fresh collagenase solution. The supernatant was saved every 15 min until all pieces were completely dissociated. The obtained cells were washed by centrifugation at 140 g for 5 min and resuspended in knock-out Dulbecco's modified Eagle's medium (knock-out DMEM; Invitrogen, UK), nonessential amino acids (GTF, Sweden), Primocin 100 μg/mL medium (Amaxa Inc., USA), 0.1 mmol/L β-mercaptoethanol (Invitrogen), glutamine 2 mM (Invitrogen), and insulin-transferrin-selenium supplement (Invitrogen). The cells were seeded directly onto plastic tissue plates (Techno Plastic Products AG, Switzerland) and cultured at 37 °C in a humidified atmosphere containing 5% CO_2, and the medium was changed twice weekly. Culturing the cells under these conditions, without adding serum to the culture medium, prohibited fibroblast growth, thus favoring cardiosphere growth.

Freezing of the cultured cardiospheres was performed by detaching the cardiospheres and resuspending in freezing medium [0.5 mL 20% knock-out serum replacement medium (Invitrogen) and 10% dimethyl sulfoxide (Sigma-Aldrich, USA) in knockout DMEM (Invitrogen)]. The cardiospheres were frozen gradually (−1 °C per minute) down to−70 °C and stored at −180 °C. When the frozen cells were recultured, they were quickly thawed to 37 °C, washed, and recultured on plastic plates.

6.4.2 Embryonic cardiomyocyte culture – Paper IV

Rat GD 11 embryos were obtained according to above and the hearts were dissected out with sharp forceps under a stereo microscope, rinsed in HBSS without Ca^{2+} and Mg^{2+} (Invitrogen, USA) and digested with collagenase type II (CLS-2, Worthington, Biochemical Corporation, USA) to produce a single cell suspension which was kept dark and on ice in culture medium containing ROCK inhibitor Y-27632 (Calbiochem, VWR Belgium). This step was repeated few times until all the tissue pieces were disassociated. The cells were spun down, resuspended in culture medium and preplated at 37 °C for 60 minutes. Attached cells were discarded and remaining cells were plated over the electrodes on the MEA culture dish in 2 mm diameter silicone wells that were removed after the cells had attached. The MEA culture dish was precoated with collagen type IV (Sigma-Aldrich, Sweden). Cells were cultured in an incubator at 5 % CO_2 and 95 % air in medium containing high glucose DMEM (GIBCO, Invitrogen, USA), primocin (Amaxa biosytems, Maryland, USA), glutamine and 2 % fetal bovine serum (FBS) (both PAA Laboratories GmBH, Pasching, Austria).

Paper V

Pregnant rats were sacrificed in a CO_2 chamber followed by cervical dislocation on GD 11 of pregnancy. Pregnant rabbits were sacrificed by boltgun followed by exsanguination on GD 11. The uterus was removed and the embryonic hearts were dissected free with sharp forceps under a stereomicroscope.

First trimester aborted human material (gestational weeks (GW) 4.5 to 10) was obtained as described under **6.4.1 Paper III**. Adult human samples were collected during open heart surgery.

Hearts were rinsed in ice cold HBSS (Hanks' balanced salt solution) without Ca^{2+} and Mg^{2+} (GIBCO, Invitrogen) and dissociated and cultured as described under **6.4.2 Paper IV** above, Culture time on glass cover slips before patch-clamp experiments was 1-3 days.

6.5 IMMUNOHISTOCHEMICAL CHARACTERISATION

6.5.1 Human cardiospheres and embryonic heart – Paper III

Hearts and cardiospheres were either paraffin-embedded or cryopreserved and sliced into 2.5-to 5- µm-thick sections by use of a microtome (Microme, HM 355S) (Microm Laborgeräte GmbH, Walldorf, Germany) or cryostat (Leica JUNG CM3000, Germany), respectively. Before the immunohistochemical staining, the paraffinembedded sections were deparaffinized and the frozen sections were fixed in 4% formaldehyde, blocked with serum, and incubated with the following primary antibodies: Isl1 (goat anti-human Isl (AF1837, R and D systems, Minneapolis, USA)); Nkx2.5 (clone 259416, R and D Systems); troponin T (ab10214, Abcam plc, Cambridge, UK), and mouse anti rat Ki67 (clone MIB-1, Dako). The sections were incubated with different fluorescence-labeled secondary antibodies: For Isl1 Alexa Fluor rabbit anti-goat antibody was used. For the other primary antibodies, we used polyclonal rabbit anti-mouse immunoglobulins/TRITC (Dako) and rabbit anti-mouse IgG /Alexa Fluor (Invitrogen). As positive controls, we used human fetal spinal cord (Isl 1), human fetal heart (Nkx 2.5), adult human atrial appendage (troponin T), and human fetal liver (Ki67) and as negative controls, we used adult human spleen.

6.6 TRANSMISSION ELECTRON MICROSCOPY OF CARDIOSPHERES – PAPER III

Spontaneously beating cardiospheres were collected with a micropipette and immediately fixed in 2% glutaraldehyde and 0.5% paraformaldehyde in 0.1 M sodium cacodylate buffer (caco)/0.1 M sucrose/3 mM CaCl2 (pH 7.4) at room temperature for 30 min followed by 24 h at 4 °C. The specimens were rinsed in 0.15 M caco containing 3 mM CaCl2 (pH 7.4) and postfixed by incubation for 2 h at 4 °C in 2% osmium tetroxide in 0.07 M caco containing 1.5 mM CaCl2. The specimens were then dehydrated in an ascending series of ethanol followed by acetone and embedded in LX-112 epoxy resin (Ladd, Burlington, VT, USA). Semithin sections (0.5 µm) were placed on glass slides, stained with toluidine blue, and examined under a light microscope. Ultrathin sections (40–50 nm) were cut and contrasted with uranyl acetate followed by lead citrate and examined using a Tecnai 10 (FEI, Eindhoven, Netherlands) transmission electron microscope set at 80 kV

6.7 CELL ELECTROPHYSIOLOGY

6.7.1 Multi-electrode array – Paper IV

The ECMs attached to the MEA culture dish (described under **6.2.3 Multi-electrode array embryo ECG – Paper IV**) and formed a beating monolayer. Drug effects were studied 48 hours after plating in a cumulative dose response protocol. For each culture, one control recording followed by subsequent recordings with increasing drug concentrations were performed. Each recording lasted for 3 minutes to allow wash in of studied drug concentration. Between the recordings solutions were changed with a pipette. Recordings were carried out at 37 °C and all solutions were kept in an incubator at 37 °C and 5 % CO_2 and 95 % air before use. Cultures that exhibited unstable rhythm or stopped beating while control values were being determined were excluded from the experiment.

6.7.2 Patch Clamp – Paper V

The electrophysiological recordings were carried out using the patch-clamp technique. An Axopatch 200B patch-clamp amplifier and pClamp software (both Axon Instruments) were used. The extracellular solution was composed of (in mM): 130 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 HEPES, and 10 glucose (pH 7.4). The intracellular solution was composed of (in mM): 135 KCl, 1 MgCl2, 10 HEPES, and 10 EGTA (pH 7.3) (all chemicals Sigma-Aldrich). The patch pipettes were made of borosilicate glass and the pipette resistance was 4–6 M Ω with the solutions used. In addition, 10 µM nifedipine, 0.5 µM tetrodotoxin, 10 µM glibenclamide, 10 µM chromanol 298B and 10 µM E-4031 (all from Sigma-Aldrich) were used in the extracellular solution depending on which channel that should be studied. Capacitance compensation was 50-80 % and the experiments were carried out at 35 °C. Tail currents were examined in Clampfit 10 (Axon Instruments) and statistically analysed in Prism 4.0 Graphpad.

6.8 ION-CHANNEL MRNA QUANTIFICATION – PAPER V

Pregnant rats were sacrificed in a CO_2 chamber followed by cervical dislocation on gestation day (GD) 11, 13, 15 or 16 of pregnancy. Pregnant rabbits were sacrificed by boltgun followed by exsanguination on GD 11, 13, 15 or 20. The uterus was removed and the embryonic hearts were dissected free with sharp forceps under a stereomicroscope.

Human embryonic hearts were acquired as described under **6.4.2 Embryonic cardiomyocyte culture – Paper V.**

Hearts used for qRT-PCR were rinsed in HBSS without Ca^{2+} and Mg^{2+}, and frozen in on dry ice for later mRNA preparation. Each analyzed sample contained pooled embryonic hearts from several dams from rat and rabbit respectively. Two samples per embryonic GD and five adult samples were analysed for rat, three samples per embryonic GD and four adult samples were analyzed for rabbit and one GW 5.5, one GW 6.5, one GW 9.5, two GW 10 and two adult samples were analyzed for human. The human samples were divided into two groups with the GW 5.5 and 6.5 hearts representing early cardiac morphogenesis (ECM) and the GW 9.5 and 10 hearts representing late cardiac morphogenesis (LCM). Human and rat adult samples were taken from the ventricle, while separate samples from atria and ventricle were analysed for rabbit.

Total RNA was extracted from embryonic and fetal samples as well as rat adult heart samples using QIAGEN Rneasy kit. Total RNA from human and rabbit adult hearts

was extracted using QIZzol (QIAGEN). The quality of the human extracted RNA was analysed using an Agilent 2100 Bioanalyzer (Agilent Technologies). The amount of total RNA was also quantified with a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies). Total RNA were subsequently reverse transcribed using High capacity cDNA archive kit (Applied Biosystems). Quantification of relative concentrations of specific mRNA sequences was performed using fluorescence-based real-time PCR on a 7900HT sequence detection system (Applied Biosystems). The designed primers and probes used in this study were synthesized by Sigma. Commercially available gene expression assays and other reagents for analysis were ordered from Applied Biosystems. The CT values (i.e. the number of cycles needed to overcome a pre-set threshold level of fluorescence) were analyzed using the SDS 2.2 software (Applied Biosystems). Each sample was analyzed as duplicates. The mean CT value for a given amplified sequence (amplicon) in a sample was subtracted from the mean CT value of the internal control gene (18S rRNA), measured in the same sample. This value is designated ΔCT. The expression level for each sample is reported as the exponential 2-ΔCT. This value was multiplied with 106 and presented as ppm of the internal control gene 18S.

6.9 STATISTICAL ANALYSIS

Paper I

A value of $p < 0.05$ was considered statistically significant. The comparison of the control group and the PHT group with respect to hypoxia/staining was performed using Kruskal Wallis one-way ANOVA followed by Wilcoxon Mann Whitney U tests. The litter was used as unit of assessment. The comparisons with respect to interval between individual heart beats were analyzed on embryoniclevel with one-way ANOVA followed by Dunnett's test.

Paper II

The comparisons with respect to intervals between individual heartbeats were analyzed at the embryonic level with one-way ANOVA followed by Dunnett's test. A value of p < .05 was considered statistically significant. In experiment 1, intervals between individual heartbeats before administration of each test compound (including vehicle-treated controls) were compared with the intervals recorded after addition of the test compound. Embryos showing severely irregular rhythm after PHT (at 200 lm), DMO (5, 10, and 20 mM), CBZ (300 lM), and PB (2,500 lM) were not included in the statistical analysis regarding interbeat intervals (see Results section for details) because it was not possible to identify peaks (individual heartbeats). In experiment 2, the interbeat intervals after addition of different combinations of AEDs were compared with those recorded after administration of individual AEDs as well as those of vehicle- treated embryos (controls). For the evaluation of the frequency of irregular rhythm between AED-treated groups and controls in experiment 1 the Kruskal-Wallis test was used in conjunction with the Mann-Whitney test (p < .05). No embryo with severely irregular rhythm was observed in experiment 2.

Paper III

For statistical analysis of the electrophysiological measurements, t tests for dependent samples were used. A P<0.05 was considered to indicate significance.

Paper IV

WEC study: For each embryo, mean IBI and QT_C from 30 consecutive complexes were calculated for each of the two measurements. Statistics were calculated on the differences between the two measurements. The values from the control groups were pooled and compared to the treatment groups with the Mann-Whitney U test.
Wilcoxon signed rank test was used to determine significance between the first and second measurement in the control group.
ECM study: The last 30 consecutive field potentials of the 3 minute recording were averaged to obtain values for IBI, FPdur, FPmin and FPmax. Average values for each concentration were compared to control with the Wilcoxon signed rank test.

Paper V

Current densities from embryonic cardiomyocytes were compared between the species using one way ANOVA followed by Dunnet's test. The same method was used to compare mRNA levels ($2^{-\Delta CT}$ values) for every gene within each of the three species, using the earliest embryonic sample as control. Statistical inter-species analysis was not performed in the mRNA study due to the large potential of error when comparing different probes and samples from different cDNA preparations. A p-value <0.05 was considered statistically significant.

7 RESULTS

7.1 PAPER I

Aims

To investigate the potential of phenytoin to induce embryonic hypoxia during the susceptible stage for induction of malformations (GD 10 in the mouse) using the hypoxia marker pimonidazole. To repeat the experiment at a later gestational age (GD 15) when I_{Kr} is no longer important for cardiac repolarization. To use the new digitalization method to characterize phenytoin-induced embryonic arrhythmia.

Results

Hypoxia probe pimonidazole

Pregnant mice were administered pimonidazole followed by phenytoin or saline at gestation day (GD) 10 or 15. Pimonidazole crosses the placenta and forms covalent adducts in cells with an oxygen tension (pO_2) lower than lower than 10 mmHg.
Phenytoin treatment resulted in significant increased hypoxia probe staining in embryonic tissue at GD 10. Average staining was 6 times higher compared to control in the phenytoin 100 mg/kg group and 11 times higher in the 150 mg/kg group (see figure 8). At GD 15 phenytoin also induced significant hypoxia but the effect did not appear to be dose-dependent and was less pronounced compared to control (3 times control) (see table 1).

Cardiac activity

The effect on cardiac rate and rhythm of phenytoin was studied in cultured GD 10 embryos. Use of the new digitalization method allowed for objective analysis of the inter-beat interval and visualisation of arrhythmias (see figure 9). Phenytoin prolonged inter-beat interval and induced arrhythmias. Doses of 0-250 µM resulted in concentration-dependent prolongation of the inter-beat interval. Bradyarrhythmia and episodes of cardiac arrest were detected in the higher dose groups (see figure 9).

Conclusions

The results support the proposed mechanism that the observed dose-dependent arrhythmogenic cardiac effects induced by phenytoin result in dose-dependent hypoxia and malformations during the period when I_{Kr} is important for cardiac repolarization, and that this mechanism is not present after this period. The new digitalization method provides an objective analysis of the inter-beat interval and allows visualization of arrhythmias.

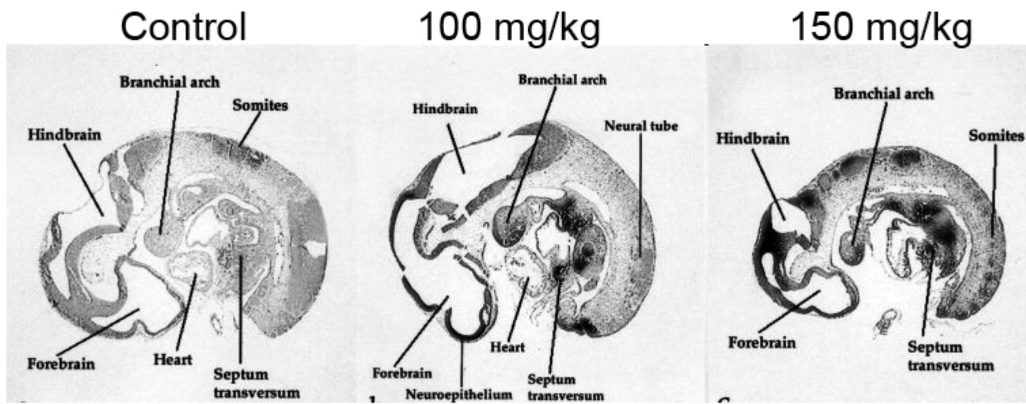

Figure 8. Dose-dependent increased staining for the hypoxia probe pimonidazole induced by phenytoin in GD 10 mouse embryos.

Table 1
Immunostaining Results (Grey Scale Units) from Treatment with Phenytoin or Tap Water (Controls) followed by the Hypoxia Marker Pimidiazole

	Day of treatment					
	Gestation day 10			Gestation day 15		
Treatment (mg/kg)	Control	Phenytoin (100)	Phenytoin (150)	Control	Phenytoin (100)	Phenytoin (150)
No. of litters	4	4	4	4	4	4
No. of embryos	14	16	15	14	14	14
Average hypoxia staining (whole embryo ± SD)	2.0 ± 0.4	13 ± 5.4[a]	23 ± 13.6[a]	8.2 ± 6.2	26 ± 9.2[a]	30 ± 8.5[a]
Ratio phenytoin/control	–	6.5	11.5	–	3.2	3.7

$^{a}p = < 0.05$.

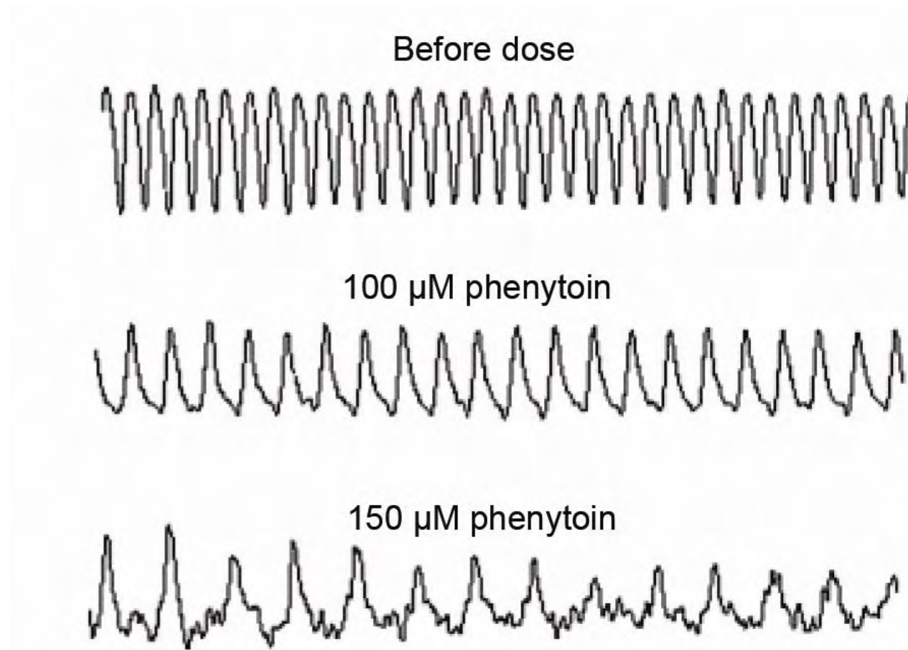

Figure 9. Concentration-dependent bradyarrhythmia induced by phenytoin in cultured GD 10 mouse embryos. Higher concentrations resulted in epoisodes cardiac arrest.

7.2 PAPER II

Aims

To investigate if the antiepileptic drugs and human teratogens phenytoin, phenobarbital, dimethadione and carbamazepine induce embryonic cardiac arrhythmia. To study if exposure to combinations of the AEDs , mimicking combination therapy, result in aggravated arrhythmogenic cardiac effects compared to exposure to a single AED.

Results

Single administration

All of the studied AEDs prolonged the inter-beat interval and induced irregular rhythm in a concentration-dependent manner (see figure 10 and 11). Irregular rhythm was induced within therapeutic concentrations for dimethadione.

Combined administration

Exposure to combinations of AEDs with phenytoin resulted in aggravated effects on inter beat interval compared to monotherapy, at clinically relevant concentrations (see figure 12).

Conclusions

The studied AEDs induce concentration-dependent embryonic bradycardia and arrhythmia. Combinations of AEDs induce embryonic bradycardia at clinically relevant concentrations and the effect is aggravated compared to single exposure. The results support the idea that the increased risk for malformations following combination therapy is linked to an increased risk for cardiac rhythm disturbances.

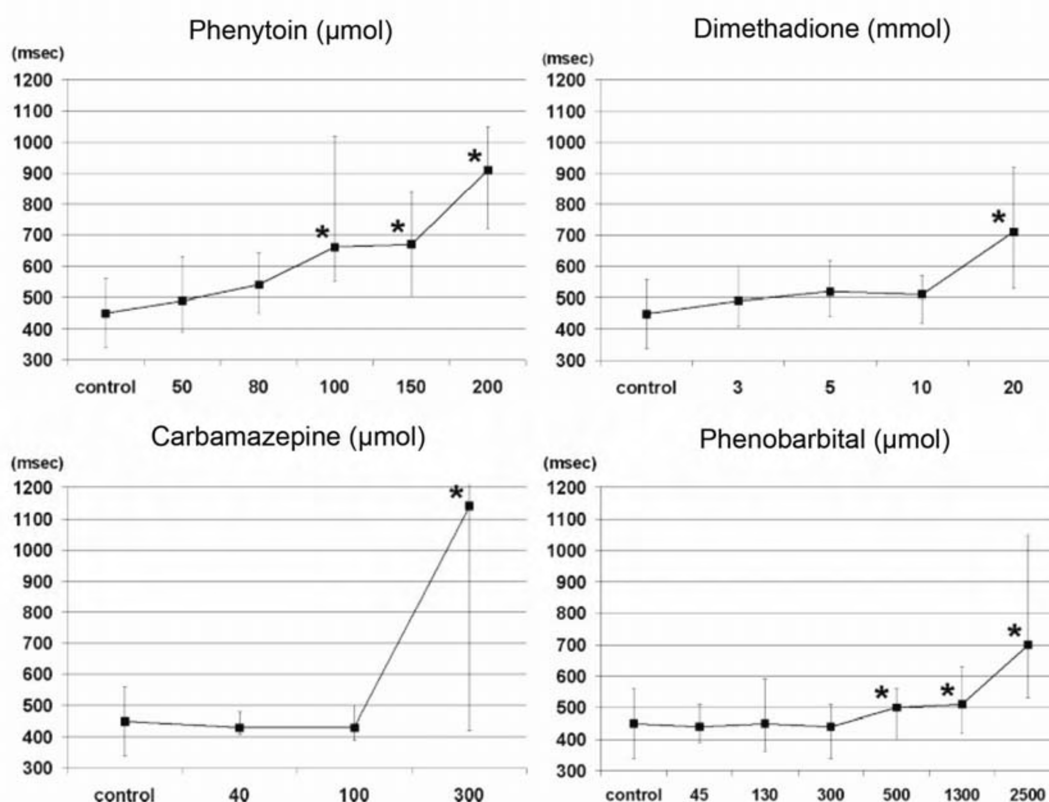

Figure 10. Concentration dependent prolongation of the IBI induced by AEDs in GD 10 cultured mouse embryos. * = p<0.05.

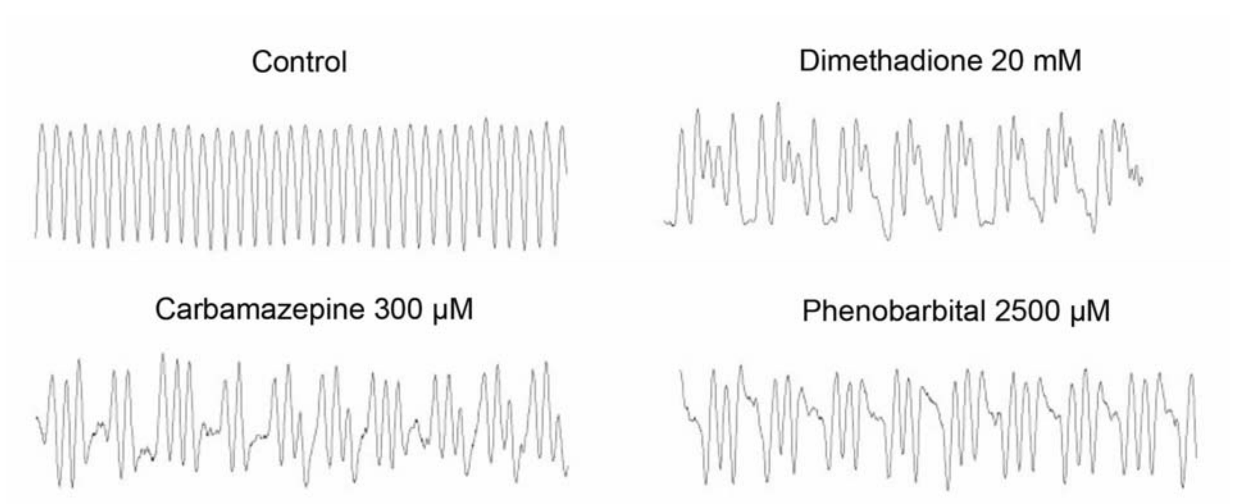

Figure 11. Irregular arrhythmias induced at above therapeutic concentrations by dimethadione, carbamazepine and phenobarbital in GD 10 cultured mouse embryos.

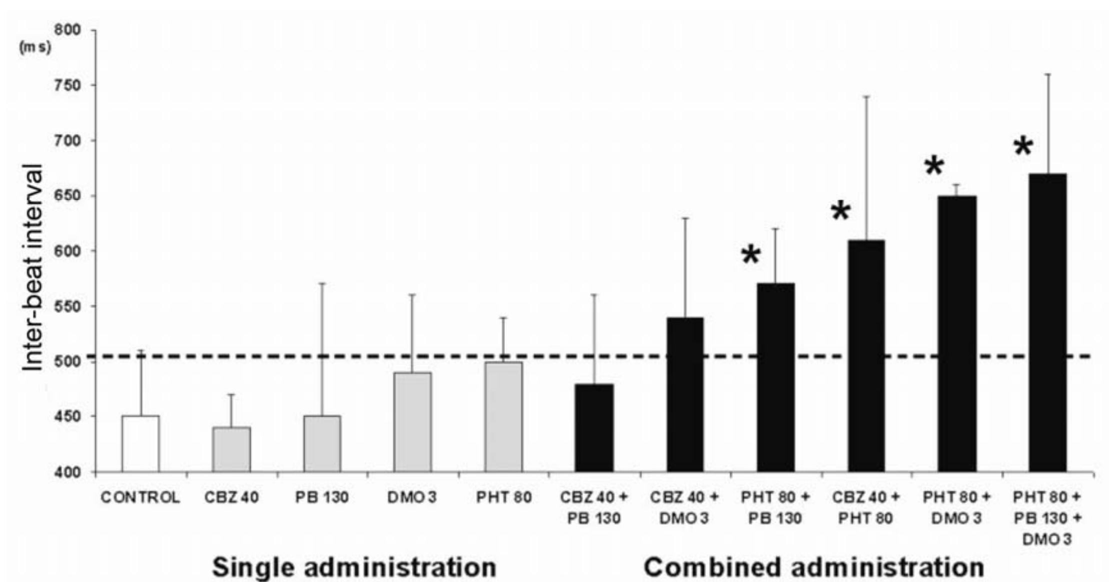

Figure 12. Significant increase in IBI induced by therapeutic concentrations of phenytoin in combinations with carbamazepine, dimethadione and phenobarbital in GD 10 mouse embryos. * = $p<0.05$.

7.3 PAPER III

Aims

To decribe the distribution of $Isl1^+$ progenitor cells and their proliferative and differentiating capacity in the early first trimester human embryonic heart. To electrophysiologically characterize beating cardiospheres derived from human embryonic hearts.

Results

$Isl1^+$ progenitor cell distribution

$Isl1^+$cells were identified to be mainly clustered in the outflow tract and to a lesser extent in the atria and in the right ventricle. Some of the clusters were also troponin T^+. Unexpectedly a only few $Isl1^+$ cells were $Ki67^+$ while in the ventricles a majority of $Isl1^-$, troponin T^+ cells were $Ki67^+$.

Cardiosphere characterization

Cultures derived from the digested embryonic heart developed into spontaneously beating cardiospheres. At harvest cells in these cardiospheres showed frequent expression of troponin T and Nkx2.5, while Isl1 was expressed only in scattered cells. Only a minority of the cultured cells expressed Ki67. The cardiospheres could be frozen, thawed, and recultured to beating cardiospheres, and electron microscopy revealed cardiomyocyte phenotype and cell-to-cell coupling (see figure 13).
Autonomous IBI and field potential duration (FPdur) were determined from extracellular multi-electrode array (MEA) recordings of cardiosphere clusters (1579 ms, SD= 633, range 923–2748 ms and 172 ms SD = 58, range 110–265 ms respectively). Electrical stimulation of a cluster of cardiomyocytes in 100 ms steps between 1000 and 600 ms showed rate dependency of the FPdur with a decrease of up to 35% at the highest stimulation frequency. β-adrenergic stimulation with 35,9µM isoprenaline significantly decreased the inter beat interval with 54% and shortened the FPdur with 23% (see figure 14).

Conclusions

$Isl1^+$ cells are present in the outflow tract, atria, and right ventricle in the first trimester human embryonic heart and have the capacity to differentiate into cardiomyocytes. Spontaneously beating cardiospheres derived from embryonic hearts display rate dependency of the action potential duration and sensitivity to β-adrenergic stimulation.

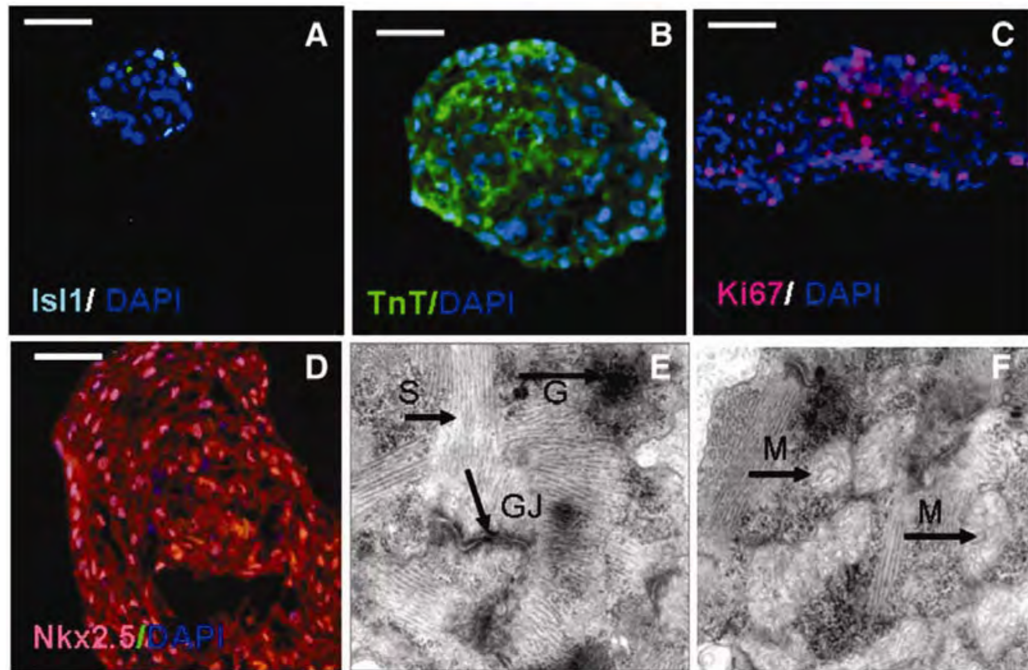

Figure 13. Characterization of beating cardiospheres. Beating cardiospheres expressed (A) Isl1, (B) troponin T, (C) Ki67, and (D) Nkx2.5. The nuclei are stained blue by DNA staining with DAPI. Bars represent 50 µm. (E and F) Under electron microscopy, it was evident that the cells in the cardiospheres represented metabolically active cardiomyocytes with sarcomeric structures (S), mitochondria (M), and glycogen deposits (G), and that they formed gap junctions (GJ) with the surrounding cardiomyocyte. The contractile elements were not organized in the cytoplasm, a feature seen in cardiomyocytes that do not perform any work.

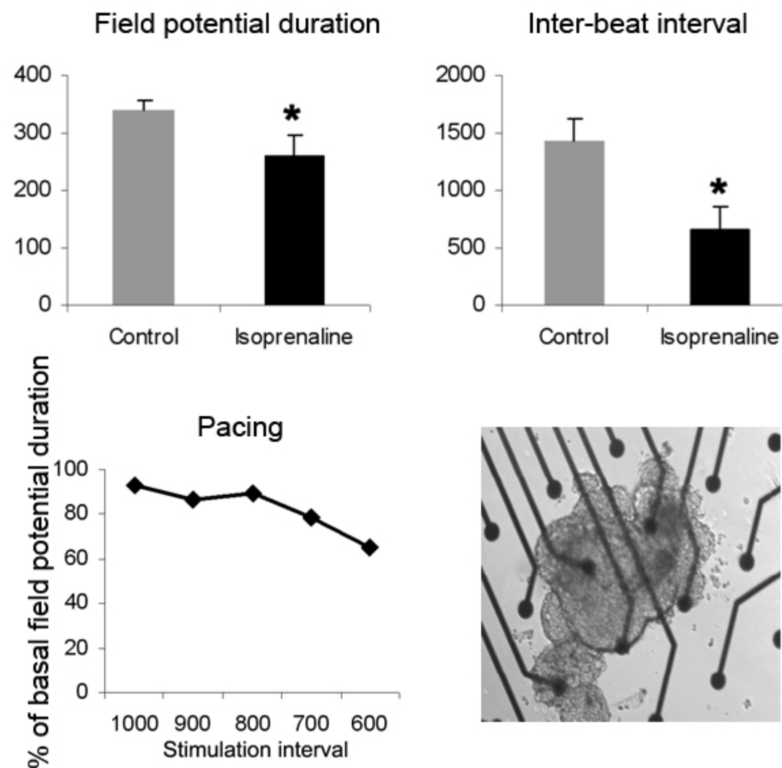

Figure 14. Cardiospheres attached to MEA culture dishes allowing electrophysiological characterization (bottom right). The beating cardiospheres displayed sensitivity to adrenergic stimulation (top) and rate dependency of the field potential duration in a pacing experiment. * = $p<0.05$.

7.4 PAPER IV

Aims

To study the electrophysiological character of the embryonic cardiac effects of phenytoin and the potent selective I_{Kr} blocking drug E4031, and investigate if the arrhythmia is associated with QT prolongation. To investigate the potential of rat ECMs to predict the arrhythmogenic effects in vivo.

Results

Embryo ECG

ECG could be obtained from GD 11 rat embryos. Pheytoin and E4031 both induced concentration-dependent bradyarrhythmia and QT_C prolongation (see figure 15). At the higher tested concentrations, phenytoin-induced cardiac arrest and E4031-induced AV-nodal block (see figure 16).

ECM electrophysiology

In the ECM model, phenytoin and E4031 both decreased IBI. Phenytoin decreased FPdur while E4031 increased FPdur in a concentration-dependent manner. At the higher tested concentrations, phenytoin induced arrested beating activity and E4031-induced irregular tachyarrhythmias and EADs (see figure 17 and 18).

Conclusions

QT_C prolongation precedes drug-induced embryonic arrhythmia. Sensitivity and the types of arrhythmias induced differ from the adult, indicating that adult data may be insufficient to determine the risk for the embryo. Rat embryonic cardiomyocytes display a similar sensitivity as the embryonic heart but respond with different arrhythmias to drug exposure and further development of the method is necessary.

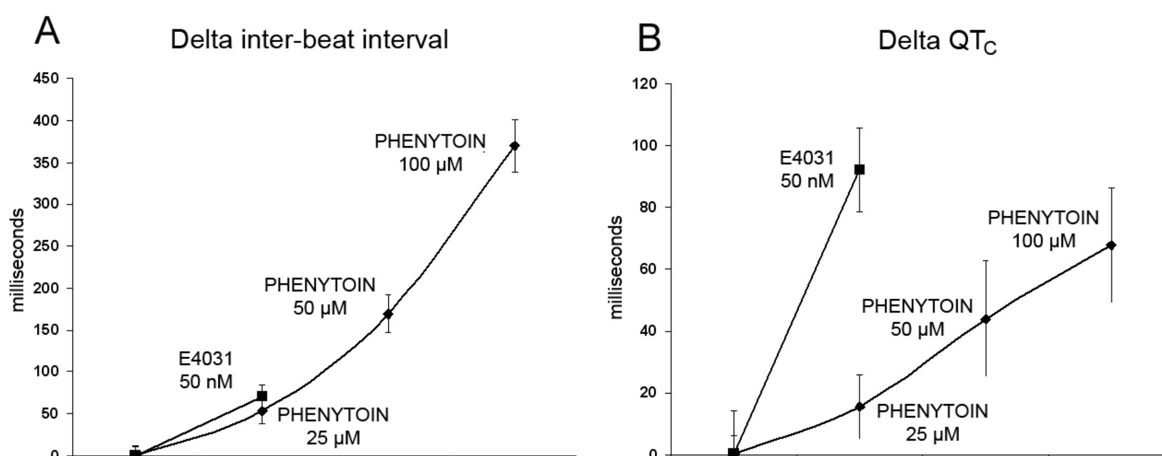

Figure 15. Phenytoin and E4031 induced concentration-dependent prolongation of IBI and QT_C in GD rat embryos. Embryos exhibiting arrhythmia or cardiac arrest were excluded from the analysis. $p<0.05$ for all values compared to control.

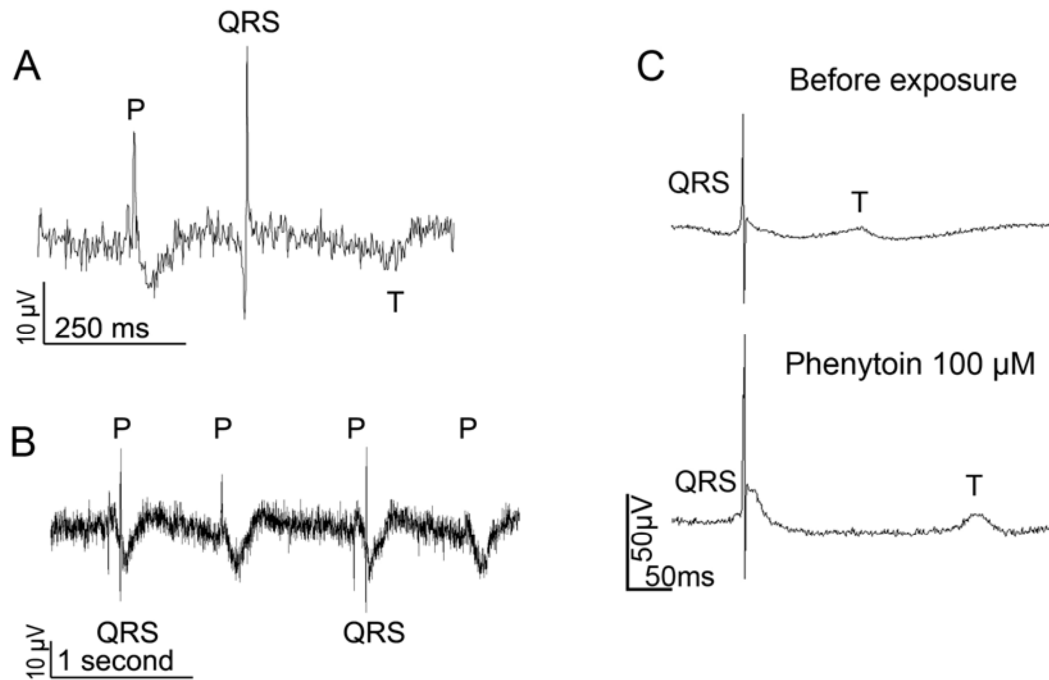

Figure 16. A. Gestation day 11 rat embryo ECG with P-wave, QRS complex and inverted T-wave indicated. B. 2:1 AV-block induced by the selective hERG blocker E4031 at 1 µM. C. Representative QT prolongation induced by 100 µM phenytoin.

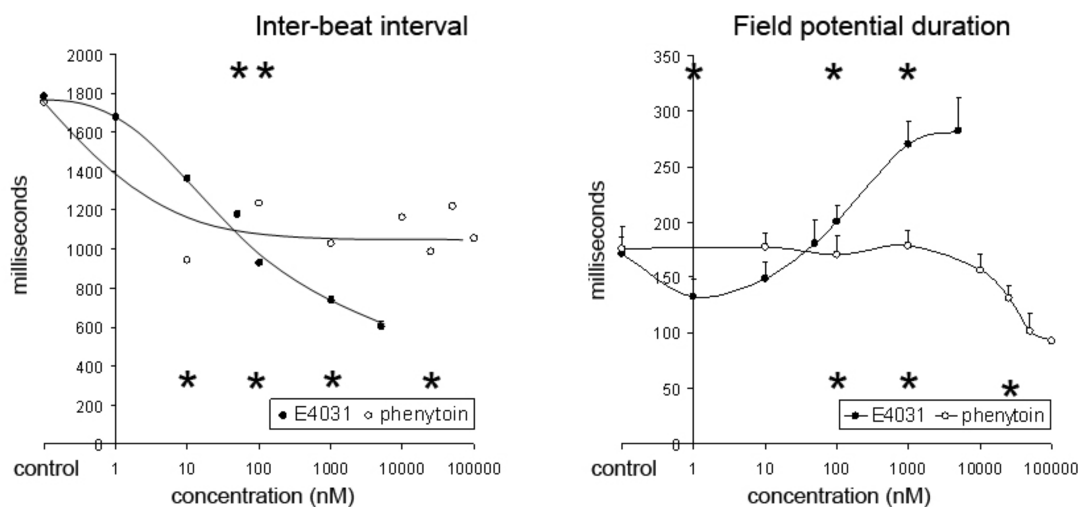

Figure 17. Phenytoin and E4031 induced a decrease in the inter-beat interval in GD 11 rat embryonic cardiomyocytes. The substances induced opposite effects on the rate corrected field potential duration. Cultures exhibiting irregularity or arrest in beating activity are excluded from analysis. * = $p<0.05$ compared to control for E4031 (top of figure) and phenytoin (bottom of figure).

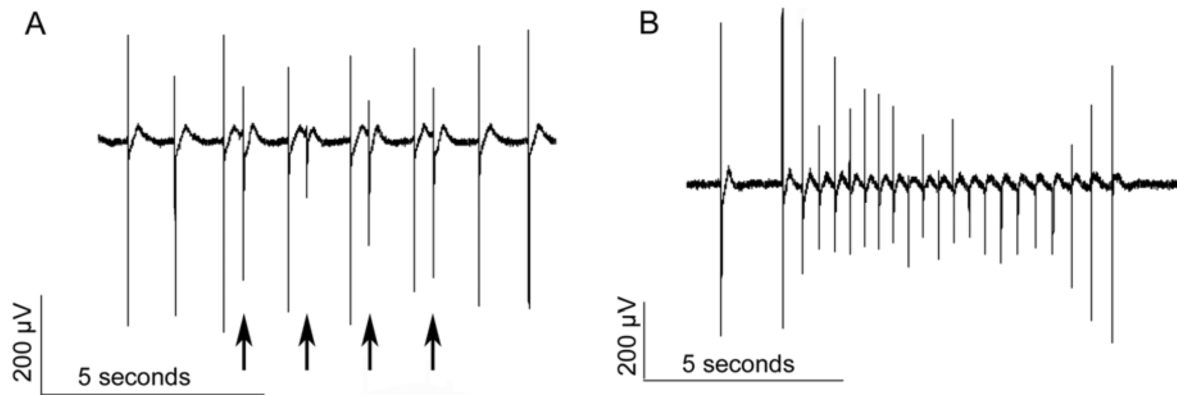

Figure 18. Representative examples of irregular spontaneous beating activity in rat gestation day 11 embryonic cardiomyocytes induced by selective hERG blocker E4031 at 5 µM. A. Early after depolarizations (EADs) indicated by arrows. B. Irregular tachyarrhythmia.

7.5 PAPER V

Aims

To compare mRNA and electrophysiological properties in the developing heart in human, rat and rabbit with focus on delayed rectifier ion channel function and to relate the findings to susceptibility to drug-induced embryonic arrhythmia.

Results

Patch clamp

The I_{Kr} current was present in cardiomyocytes from gestation day 11 rat and rabbit and gestation week 4.5 human embryos. In the human the current density was ~50% lower compared to the other species. Presence of I_{Ks} (not studied in human) was found in rat but not in rabbit cardiomyocytes (see figure 19)

Quantitaive real-time PCR

Transcript levels of the I_{Kr} related subunit Kv11.1 revealed that transcription of Kv11.1 was present in all species and underwent changes during embryonic cardiac development. Both Kv7.1 and MiRP1 displayed species differences indicating that I_{Kr} is down-regulated in late embryonic life in the rat.

Transcript levels of the I_{Ks} related subunits, pore forming Kv7.1 and ancillary β-subunits, MinK, MiRP2 and MiRP3 also displayed clear species differences. Expression patterns of I_{Ks} related transcript levels were low in the rabbit, and increased in the rat during cardiac development see (figure 20).

Conclusions

The I_{Kr} current is expressed and functional in the embryonic heart in species used in teratology testing (rats and rabbits) as well as in the human embryo, but may lose importance at the end of the embryonic period in the rat. I_{Ks} appears to provide additional repolarization reserve in human and rat embryos, but is not present in the rabbit. These differences in delayed-rectifier channel density indicate that the human and rabbit embryo may be more susceptible to drug-induced embryonic cardiac rhythm disturbances compared to the rat. These results give further evidence of human relevance for the I_{Kr} -related teratogenic mechanism observed in animal studies.

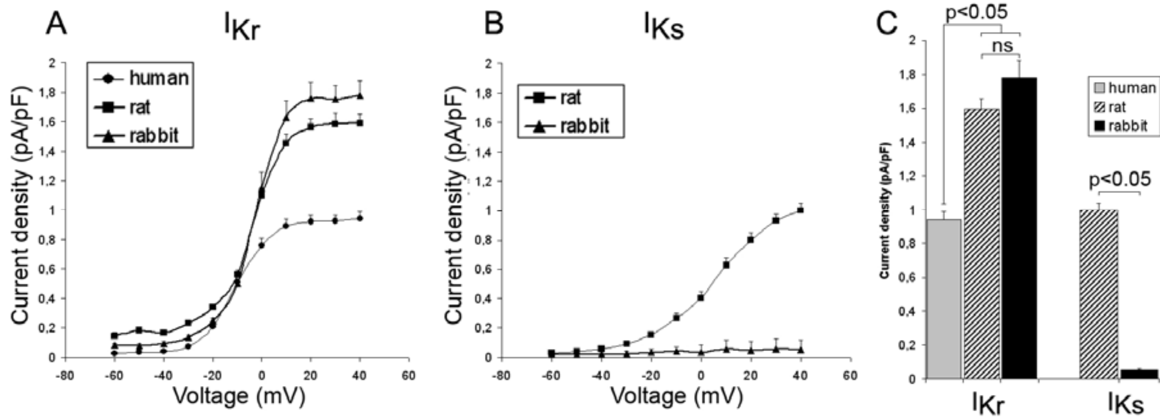

Figure 19. Whole cell patch clamp recordings of delayed rectifier components I_{Kr} and I_{Ks} in embryonic cardiomyocytes (human gestational week 4.5, rat and rabbit gestational day 11). Tail current densities of I_{Kr} (A) and I_{Ks} (B) elicited after return to –60 from depolarizing test pulses. (C) Current densities at +40 mV compared between species. I_{Ks} was not studied in human. Values presented as average pA/pF + SEM. Number of cells analyzed were for I_{Kr}: human 6, rat 9 and rabbit 8, and for I_{Ks}: rat 7 and rabbit.

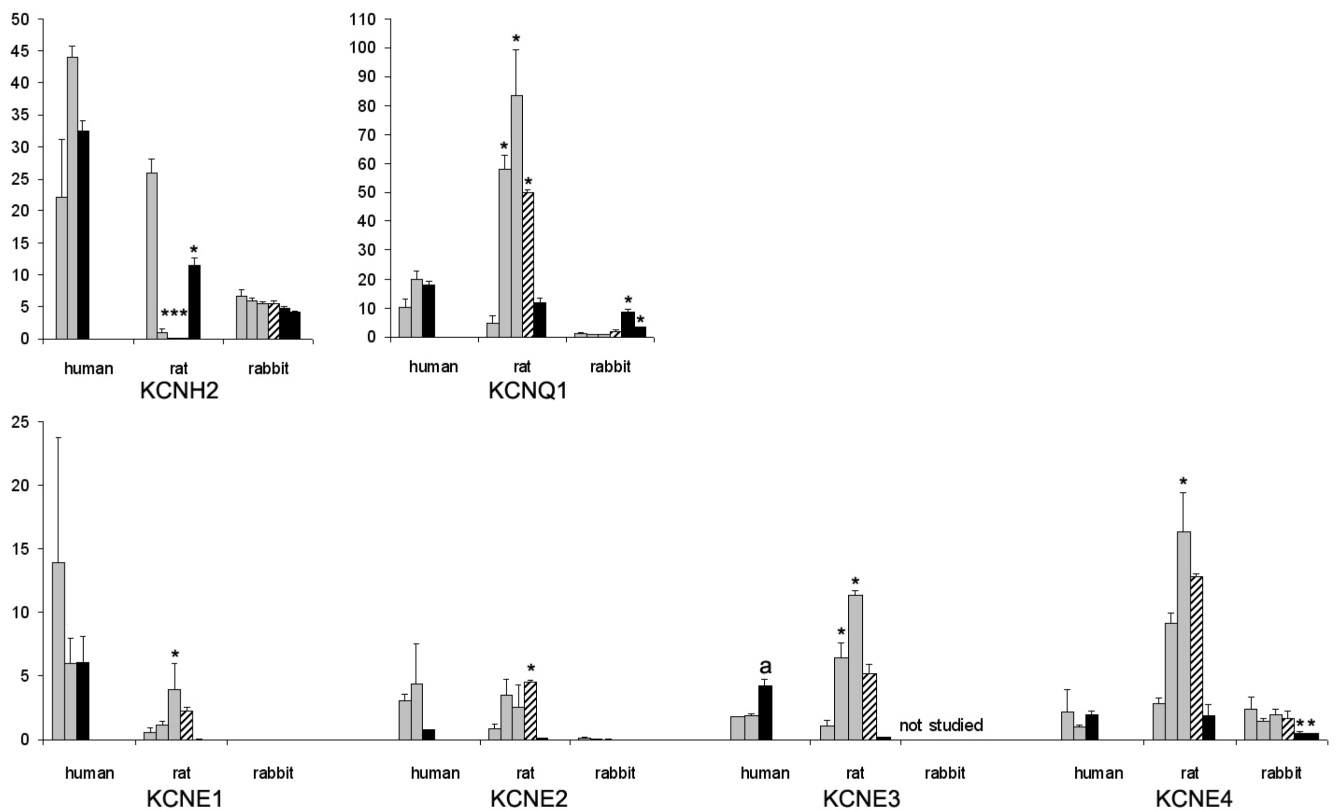

Figure 20. Transcription levels of delayed rectifier channel coding mRNA .Y-axis represents relative transcription levels expressed in ppm of 18S, values are mean + SEM. Embryonic samples (grey bars) are early and late cardiac morphogenesis (GW 5.5-6.5 and 9.5-10 respectively) in human samples and GD 11, 13 and 15 in rat and rabbit samples. Striped bars represent fetal samples (GD 16 in rat and 20 in rabbit) and black bars adult samples. In rabbit adult samples atria (left bar) and ventricle (right bar) are displayed separately. * = $p<0.05$ compared to the earliest embryonic sample, a = significant compared to late cardiac morphogenesis sample.

8 GENERAL DISCUSSION

8.1 DOES PHENYTOIN INDUCE EMBRYONIC HYPOXIA?

Hypoxia in embryonic development appears to be an important regulator of growth and differentiation. The hypoxia probe pimonidazole has been used to show that the oxygen tension in several tissues is below 10 mmHg during mouse embryonic development (Lee *et al.*, 2001). In the mouse embryo hypoxia promotes chondrocytic differentiation and cartilage matrix synthesis and suppresses terminal chondrocyte differentiation, enhancing and preserving their phenotype and function during chondrocyte differentiation and endochondral ossification (Hirao *et al.*, 2006). Hypoxia also appears to play an important role in vascular development in the embryo. HIF1α, VEGF and PECAM co-express with hypoxic regions indicating vascular development and endothelial cell proliferation (Lee *et al.*, 2001). Recently, hypoxic signalling via HIF1α has also been shown to be essential for normal cardiac development in mice (Krishnan *et al.*, 2008). However, as described in the introduction, too much hypoxia, induced by uterine vessel clamping or arrhythmia following selective I_{Kr} blockers, is associated with a specific set of malformations (including stage specific skeletal defects, distal digital reductions, cleft lip and palate as well as cardiovascular anomalies). In the rat, the selective I_{Kr} blocker almokalant induces embryonic bradyarrhythmia and hypoxia, shown by increased staining for pimonidazole, on GD 11 and 13 but not on GD 16, suggesting a close correlation between the period where I_{Kr} is functional and the teratogenic potential of almokalant (Danielsson *et al.*, 2003b). In paper I, we set out to investigate if phenytoin has the potential to induce embryonic hypoxia in the mouse. The results show that dose-dependent increased staining for pimonidazole can be induced on GD 10 (phenytoin 100 µM: 6 x control and 150 µM: 11 x control). This result supports the idea of a common arrhythmia related teratogenic mechanism for I_{Kr} blocking drugs and phenytoin. There was also an increase in staining for pimonidazole on GD 15, a time in development when I_{Kr} has lost importance for cardiac repolarization. The increase compared to control was however half of the observed increase on GD 10, and did not exhibit dose dependency (~3,5 x control for both concentrations). This finding is in contrast to the observed effects of selective I_{Kr} blocker almokalant, and suggest that phenytoin-induced hypoxia is not only a result of arrhythmia following I_{Kr} inhibition, but also other ion channels such as Na^+ and Ca^+.

8.2 PHARMACOLOGICAL EFFECTS ON THE EMBRYONIC HEART

8.2.1 Rate and rhythm

The antiepileptic drugs phenytoin, dimethadione, phenobarbital and carbamazepine have all been associated with a similar range of malformations in humans and animals (Dansky & Finnell, 1991). They also inhibit hERG in a concentration-dependent manner (Danielsson *et al.*, 2003a). As described in the introduction, a common teratogenic mechanism involving embryonic arrhythmia-induced hypoxia, has been proposed for these AEDs. The effects on the embryonic heart induced by dimethadione and phenytoin have been investigated in previous studies (Azarbayjani & Danielsson, 2001, 2002). Both compounds induce concentration-dependent arrhythmias and cardiac arrest in vitro and in vivo. In paper I and II we wanted to investigate further if the pharmacological effects on rate and rhythm support the proposed mechanism. We utilized a newly

developed method for digitalization of cardiac activity to study the effects of phenytoin, dimethadione, phenobarbital and carbamazepine with higher precision than in previous whole embryo culture experiments (Skold *et al.*, 2002). The computer automated determination of IBI increased reproducibility of analysis and eliminated the risk of biased interpretation. The new method also enabled visualisation of arrhythmias and the possibility of inter-treatment comparison. The effects of increasing concentrations of the AEDs were studied in C57BL/6J mouse embryos. Concentrations were chosen to start at within the recommended therapeutic interval. All AEDs induced bradycardia and irregular tachyarrhythmias in a concentration-dependent manner. In the statistical analysis bradycardia was observed above therapeutic concentrations. In hindsight, the choice of high end concentrations of carbamazepine (therapeutic range 20-40 µM), proved insufficient to illustrate a concentration-response curve, since no effect was observed at 100 µM and a severe effect on rate and rhythm was induced at 300 µM. Dimethadione, induced irregular tachyarrhythmias at 5 and 10 mM (therapeutic range 3 – 10 mM) in 20% of the embryos, while inducing a slight, in our study not statistically significant, bradycardia in remaining embryos. This suggests that slight bradyarrhythmias detected within the short observation window of our method, may be associated with risk of more severe tachyarrhythmias if exposure occurs throughout pregnancy. We can speculate that the bradycardia preceding irregular arrhythmias is associated with prolongation of cardiac repolarization as was observed with almokalant (Abrahamsson *et al.*, 1994). In all, the similarity in response to increasing concentrations of different AEDs support a common arrhythmia related teratogenic mechanism.
Polytherapy during pregnancy with combinations of the above mentioned AEDs is associated with increased risk of major malformations, compared to monotherapy (Dansky & Finnell, 1991; Pennell, 2003). In the second part of the study in paper II we aimed to mimic the effect of polytherapy on the embryonic heart. Cultured embryos were exposed to combinations of phenytoin, dimethadione, phenobarbital and carbamazepine at clinically relevant concentrations. The results show an additive prolonging effect on the IBI of the AEDs. Phenytoin in different combinations with the other AEDs, induced statistically significant prolonagation of the IBI. This finding, indicating an increased long term risk of arrhythmia, provides a plausible explanation to why polytherapy is more potent than monotherapy at inducing terata. We aimed to replicate the in vivo situation in our experimental setup. In the clinical situation, the AEDs are protein bound to different degrees and the culture medium in this study contained mainly heat inactivated rat serum. We can speculate that competitive protein displacement may add to the increased observed effects after combined exposure in vitro and in vivo. However, other metabolic considerations such as protein displacement effects on availability for clearance and pharmacological induction of metabolizing enzymes could not be recreated in this setup.

8.2.2 Embryonic ECG

In adult humans selective I_{Kr} blockers can induce bradycardia, QT-prolongation, EADs and Torsades de Pointes arrhythmias. Phenytoin can also induce bradycardia, but is not associated with QT prolongation, TdP or other ventricular tachyarrhythmias (Redfern *et al.*, 2003). The embryonic heart cannot however be expected to respond in same way as the adult heart to ion channel inhibition. As described in the introduction, factors such as cardiac morphology, innervation, expression and regulation of ion channels as well

as formation of the conduction system may all influence sensitivity to pharmacologically-induced arrhythmia, although little is known about their individual importance. In the rat embryo selective I_{Kr} blockers appear to induce similar cardiac pathology as in the adult human, including APD prolongation, AEDs and arrhythmia (Abrahamsson *et al.*, 1994). The electrophysiology of phenytoin effects on the embryonic heart has however not been studied and the results in paper I and II raise several questions regarding the mechanisms behind the observed drug-induced arrhythmias. For instance, it is not known if the observed bradycardia is associated with pathological prolongation of cardiac repolarization, which has been proposed previously (Skold *et al.*, 2002). Further, when comparing the digitally visualized bradyarrhythmias induced by phenytoin with the more potent I_{Kr} blocker cisapride (Skold *et al.*, 2002), it appears that they differ in degree of irregularity, and phenytoin did not induce the irregular tachyarrhythmias that have been observed after exposure to selective I_{Kr} blockers.
In an attempt to answer and explain these questions and observations, we developed a novel method acquiring embryonic ECG. Whole embryo culture was carried out in multi-electrode array culture dishes and ECG was obtained before and after exposure to phenytoin. In order to study the effects of a selective I_{Kr} blocker more in detail, as well as provide a basis for comparison with phenytoin, the potent I_{Kr} blocker E4031 was also tested.
The results showed that exposure to phenytoin resulted in concentration-dependent bradycardia and QT_C prolongation. At the higher tested concentrations cardiac arrest was induced. Exposure to the selective hERG blocker E4031 resulted in bradycardia, QT_C prolongation and with increasing concentrations AV block and irregular ventricular arrhythmias.
These data support the suggested association between prolongation of IBI and prolongation of cardiac repolarization and arrhythmias induced by carbamazepine, phenobarbital and dimethadione in paper II, as well as cisapride in a previous study (Skold *et al.*, 2002). Similarly to the adult heart, our data suggest that QT_C prolongation can be used to predict risk for arrhythmia in the embryo. However, the sensitivity of the embryo to react with QT_C prolongation and arrhythmia, as well as the types of arrhythmias observed, were different compared to what is known about the adult human. Neither AV block induced by potent selective I_{Kr} blockers nor phenytoin-induced QT_C prolongation are known side effects in human adults. These differences in drug response indicate that adult data may not be applicable to predict the risk for embryonic arrhythmia by drugs acting on ion currents of importance for cardiac repolarisation. Whole embryo culture with ECG may provide such a model.
The differences in induced arrhythmias between the pure I_{Kr} blocker E4031 and phenytoin, which inhibits several cardiac ion currents (IC50: 50 µM, 200 µM and 242 µM for Na^+, Ca^+ and I_{Kr} respectively (Yatani *et al.*, 1986; Barber *et al.*, 1991; Danielsson *et al.*, 2003a) provides evidence that embryonic arrhythmogenicity by phenytoin is caused by inhibition of a combination of cardiac ion currents. This suggests that the type of arrhythmia, whether it is bradycardia, periods of cardiac arrest, AV block or irregular tachyarrhythmias, is of subordinate importance for the teratogenic potential, as long as the disruption of circulation results in hypoxia.

8.3 ELECTROPHYSIOLOGY OF EMBRYONIC CARDIOMYOCYTES

Safety regulations concerning drug interactions with cardiac repolarization, and the I_{Kr} current in particular, are rigorous. Preclinical (ICH_S7B, 2005) and clinical follow-up (ICH_E14, 2005) guidelines for pharmaceutical development require the potential of new compounds to block I_{Kr} /hERG, prolong APD or QT and induce arrhythmias to be thoroughly tested. A recent review highlights the magnitude of the problem by presenting hERG/I_{Kr} block data of 234 drugs and compounds (Polak *et al.*, 2009). Methods used in these studies include ECG, patch clamp on cardiomyocytes and heterologously hERG expressing oocytes, Langendorff perfused whole hearts and dissected Purkinje fibers. These models have their strengths and weaknesses. The semi-automated methods are fast and require little resources, but generally provide less information and may produce false positives. The more complex methods provide better predictive information but are slow and costly. In an attempt to bridge this problem Meyer et. al. suggested extracellular multi-electrode array recordings from spontaneously beating clusters of embryonic chicken cardiomyocytes as an alternative adult QT screening assay (Meyer *et al.*, 2004). The duration of the acquired field potentials from cardiomyocyte MEA recordings have been shown to closely correlate to the APD (Halbach *et al.*, 2003) and can therefore be used to predict QT effects.

Adopting this line of thought, we explored if the changes in rate and rhythm observed in the whole embryo culture with ECG experiments could be replicated and predicted in an embryonic cardiomyocyte assay. Culturing GD 11 rat cardiomyocytes on the MEA is less resource demanding and uses fewer animals compared to WEC studies, and could therefore be more suitable for larger scale screening. In paper IV ECMs were exposed to phenytoin and E4031 and electrophysiological recordings were carried out in the MEA system. Exposure to E4031 resulted in a decrease in IBI and with increasing concentrations EADs or irregular tachyarrhythmias were observed. Repolarization, measured as FPdur and corrected for rate, was prolonged in a concentration-dependent manner at concentrations above 100 nM. ECMs exposed to phenytoin also displayed a decrease in IBI but in contrast to E4031 exposure, FPdur was shortened. At higher concentrations arrest of beating activity occurred.

Our results show that the ECM model failed to accurately replicate the results seen in the WEC model. The types of arrhythmia observed differed and the effect on IBI was opposite to that of the whole embryo culture model. There were however also similarities in response. The sensitivity to arrest of beating activity induced by phenytoin appears to be similar and repolarization was prolonged by E4031 in both models.

The observed differences may be multifactorial in origin. The embryonic heart has a primitive conduction system which may react differently to drug exposure than the ECMs in culture, resulting in conflicting effects. In addition, cell-to-cell contact architecture and interaction differ between the two models. A recent study comparing the effect of the selective I_{Kr} blocker dofetilide in the rabbit Purkinje-fiber model and the Langedorf perfused heart model (Roche *et al.*, 2010) show results comparable to ours. In their study the sensitivity of the multicellular and the whole heart model are similar, but the whole isolated heart, with its preserved complexity, is a better model for predicting arrhythmogenicity, at least when associated with I_{Kr} /hERG inhibition. In line with these results, we can conclude from our data that the WEC with ECG model appears to be a better predictor of arrhythmogenicity, but that there are similarities in sensitivity between the two models. The ECM model however, requires further develop-

ment. Possibly a higher degree of predictability could be achieved through the analysis of other electrophysiological parameters. When attempting to predict I_{Kr} /hERG related arrhythmogenicity for adults several other biomarkers than QT prolongation have been used for risk assessment. These include action potential temporal instability, triangulation, and reverse use-dependence (the APD prolonging effect diminishes with increased rate) (Hondeghem *et al.*, 2001). It is however not known if these parameters can be translated to field potential analysis. Future transmembrane electrophysiological recording studies on ECMs may reveal effects of phenytoin and selective I_{Kr} blocker on these parameters.

8.4 IS THE TERATOGENIC MECHANISM OF HUMAN RELEVANCE?

Phenytoin is associated with a similar range of malformations across species, including humans. Our and previous animal data provide support for a arrhythmia/hypoxia related mechanism for phenytoin, as well as other AEDs and selective I_{Kr} blockers. Very little is however known about the human embryonic heart regarding electrophysiological development and sensitivity to pharmacologically-induced arrhythmia. In paper III and V we study the human embryonic heart in order to provide information about the human relevance of the I_{Kr} /hERG related teratogenic mechanism.

In paper III, we derived beating cardiospheres from human embryonic hearts and characterized them regarding maturity of phenotype and electrophysiological properties. The spheres showed a high cardiomyocyte content, rich in troponin T^+ contractile elements, mitochondria and gap junctions, suggesting functional electrically coupled contractile capacity. Cardiac developmental markers Nkx2.5, and in one cardiosphere Isl1, as well as proliferative marker KI67 could be detected, confirming that the cells were not fully differentiated. Other cardiospheres were cultured in MEA dishes and displayed sensitivity to adrenergic stimuli with isoprenaline, and rate dependency of the FPdur in a pacing experiment. These results provide a first insight into human cardiomyocyte electrophysiology, and may provide a basis for the further development of a human cardiomyocyte assay for embryonic arrhythmogenicity.

Also in paper III, we characterize the distribution, proliferation, and differentiation of $Isl1^+$ progenitor cells. If successfully expanded, these cells may provide a source of cardiomyocytes to be used in cardiac repair, or a model for pharmacological toxicity.

As described in the introduction, the electrophysiology of early embryonic cardiac development is poorly understood in humans and common species used in teratology testing (rat and rabbit). In paper V, we compare delayed-rectifier potassium channel function and expression in embryonic heart and cardiac myocytes from rabbit, rat and human at different points of cardiac development. In the first part of the study embryonic cardiomyocytes from all three species (GD 11 for rat and rabbit, GW 4,5 for human) were studied with the whole cell patch clamp method. Our results show that I_{Kr} is present in all species, and that the current density in human is approximately half the size of the other species. Expression at this time in gestation is consistent with the start of the sensitive period during which embryonic arrhythmia and embryotoxicity can be induced by I_{Kr} inhibitors in rat and rabbit (Spence *et al.*, 1994; Wellfelt *et al.*, 1999; Skold *et al.*, 2001). The results are also in line with findings in mouse were I_{Kr} is functional at this stage and where I_{Kr} inhibition also induces embryotoxicity (Davies *et al.*, 1996; Danielsson *et al.*, 2007). The finding that the I_{Kr} density is lower in human compared to rat and rabbit, indicates that other currents also are important for repolarization. Further

characterization of other currents is needed to identify which ones they are. We further report that I_{Ks} is present in rat cardiomyocytes at GD 11. Contribution to the repolarizing capacity by I_{Ks} may serve to protect the rat heart against I_{Kr} inhibition-induced arrhythmia. In rabbit however, the absence of I_{Ks} at GD 11 may indicate increased sensitivity, and is consistent with the adult situation. These data correlate well with results in teratology studies for I_{Kr} blockers, which indicate that fetal adverse effects are generated at lower doses/exposures in rabbits, compared with rats.

In the second part of paper V, we used quantitative real time PCR to study mRNA expression of delayed rectifier current subunits in human, rat and rabbit cardiac tissue at different points of gestation as well as in adult samples. Of greatest interest of the studied genes is KCNH2 (hERG). The results show that expression differs in important aspects between the three species. Most interestingly, the expression in rat was down-regulated to almost undetectable levels between GD 11 and 15. In human and rabbit samples expression was roughly equal in the embryonic samples. This finding may provide part of the explanation to why the dose threshold for I_{Kr} inhibition-induced embryotoxicity increases dramatically in the rat between GD 13 and 15 (Webster *et al.*, 1996). The half-life of the KCNH2 channel is approximately 16 hours (Zhang *et al.*, 2006), thus a down-regulation between GD 11 and 13 should result in electrophysiological changes up to two days later. Our findings are however in conflict with reported data from in situ hybridization of KCNH2 in embryonic rat hearts, where transcripts of KCNH2 are detected throughout the heart at GD 14.5 and 16.5 (Chun *et al.*, 2004). The discrepancy may be due to the in situ hybridization method not being a quantitative method. In all, the results suggest a greater dependency on KCNH2 of the human and rabbit heart for repolarization in late embryogenesis compared to rat.
We also studied the expression of KCNE2, coding for the ancillary β-subunit MiRP1. It regulates KCNH2 channel function in vitro and loss of function-mutations in KCNE2 are associated with decreased potassium flux, long QT syndrome (LQTS) and increased sensitivity to pharmacologically induced ventricular arrhythmias in adult humans (Abbott *et al.*, 1999; Sesti *et al.*, 2000). Expression of KCNE2 varied in humans and rats, and was low in rabbit samples. We can speculate that the observed normal developmental changes in KCNE2 expression alter I_{Kr} properties and possibly also sensitivity to drug-induced arrhythmia.
The I_{Ks} current is carried by the products of the KCNQ1 and the KCNE1 gene (Takumi *et al.*, 1988). Mutations in both genes are associated with LQTS (Webster & Berul, 2008). An intricate regulation of this complex by other KCNE family products probably takes place in vivo (Lundquist *et al.*, 2005; Abbott *et al.*, 2007). KCNE3 accelerates activation and mutations are associated with LQTS (Mazhari *et al.*, 2002; Ohno *et al.*, 2009) while KCNE4 inhibits I_{Ks} in vitro but the in vivo significance is not yet known (Grunnet *et al.*, 2002).
In our study KCNQ1 expression was present in the human samples during embryogenesis, and a > 10 fold up-regulation was seen in the rat. In the rabbit, KCNQ1 expression was low in the embryo and fetal samples and increased in adult samples. Expression patterns of KCNE family members (E1, E3 and E4) also differed between gestational ages and species. As mentioned earlier the interactions between the ancillary subunits are many and not fully mapped out. Species differences may occur also at the level of subunit interaction. However, the results show that KCNQ1 and KCNE1 are both expressed in embryonic life in humans and rats providing the basic condition for

I_{Ks}. This is in line with our electrophysiological findings in rat. In addition, relative levels of I_{Ks} associated transcripts are high in the rat, possibly indicating increasing importance of this current during embryonic cardiac development. In the rabbit, expression of KCNQ1 was low, KCNE1 very low and KCNE4 low. These findings suggest repressed I_{Ks} function in line with the electrophysiological data.

The results from paper V show that the I_{Kr} current is expressed and functional in the embryonic heart in species used in teratology testing (rats and rabbits) as well as in the human embryo, but may lose importance at the end of the embryonic period in rat. I_{Ks} appears to provide additional repolarization reserve in human and rat embryos, but is not present in the rabbit.

Together, these differences in delayed-rectifier channel density indicate that the human and rabbit embryo may be more susceptible to pharmacologically-induced embryonic cardiac rhythm disturbances compared to rat. These results give further evidence of human relevance for the I_{Kr} -related teratogenic mechanism observed in animal studies.

8.5 OTHER PROPOSED MECHANISMS OF PHENYTOIN TERATOGENICITY

Phenytoin-induced teratogenicity has been studied for more than 40 years. The proposed teratogenic mechanism studied in this thesis, initially presented in a paper by Danielson (Danielson *et al.*, 1992) is the latest attempt to explain this phenomenon. The other main theories that have been proposed are disruption in folate metabolism (Netzloff *et al.*, 1979), CYP450 bioactivation of phenytoin to a reactive epoxide (Harbison, 1977; Martz *et al.*, 1977) and an alternative bioactivating pathway involving the co-oxidation of phenytoin to free radical intermediates centred in the hydantoin nucleus (Wells & Vo, 1989).

The theory that phenytoin teratogenicity is mediated through interruption of folate metabolism originated from observed similarities in human malformations after maternal exposure to folate antagonists or phenytoin. However, in studies trying to reduce the teratogenicity of phenytoin by supplementation of folate no protection was observed. Instead, the teratogeninc effects of phenytoin were potentiated (Schardein *et al.*, 1973; Mercier-Parot & Tuchmann-Duplessis, 1974). The results are unexplained but do not support the theory that phenytoin teratogenicity is a result of disrupted folate metabolism.

The predominant proposed mechanism is bioactivation of phenytoin in some form. Some suggest that phenytoin is metabolized by CYP450 enzymes to an epoxide metabolite. However, studies show that none of the major metabolites induce malformations at doses where phenytoin is teratogenic (Harbison & Becker, 1974). Fluconazole-induced CYP450 inhibition has been shown to potentiate phenytoin teratogenicity, also arguing against a CYP450-dependent mechanism (Tiboni *et al.*, 2003). In addition, the CYP450 activity in the embryo during the sensitive days to phenytoin teratogenicity is very low, indicating low capacity of forming epopxide metabolites (Winn & Wells, 1995) and it is unlikely that the highly reactive epoxides produced in the maternal liver can pass the placenta.

Another suggested path of phenytoin bioactivation is co-oxidation of phenytoin to ROS intermediates by prostaglandin synthetase, capable of ROS related developmental defects as described in the introduction. The evidence for this theory does not contradict the proposed arrhythmia/hypoxia mechanism. Inhibition of mixed metabolizing path-

ways of phenytoin results in slowed metabolism, higher plasma concentrations and increased severity of malformations. The results have been explained as an increased opportunity for more of the compound to oxidize to ROS metabolites, resulting in the increased observed teratogenicity (Wells *et al.*, 1980). These data can however also be explained by increased concentration-dependent adverse cardiac effects resulting in malformations as explained in the introduction. We find the arrhythmia related mechanism a far more likely explanation to observed teratogenic effects. A wide range of drugs, including AEDs, selective I_{Kr} blockers and prokinetic gastrointestinal agents induce the same stage specific malformation as phenytoin in animal studies, but do not metabolize into reactive metabolites. They do however share the ability to induce embryonic arrhythmia with phenytoin.

8.6 FUTURE CONCEPTS

In this thesis aspects of selective I_{Kr} blockers and phenytoin effects on the embryonic heart have been investigated. We have characterized pharmacologically-induced embryonic arrhythmias and shown that this mechanism most likely is of human relevance due to conserved dependence on I_{Kr} for cardiac repolarization across species.

As mentioned previously more than 230 drugs have I_{Kr}/hERG blocking potential. Our data suggests that the tests used to predict I_{Kr} /hERG related arrhythmogenicity in adults may not be applicable to the embryonic situation due to differences in sensitivity and arrhythmias observed. Interactions with other ion currents involved in maintenance of normal cardiac activity maybe crucial to arrhythmia related teratogenicity, as appears to be the case with phenytoin, and are at risk of being missed in conventional electrophysiological models.

It has been shown to be difficult to assess teratogenic risk of drugs with I_{Kr} blocking potential both in clinical and animal teratology studies. Conventionally designed, repeated dosing, teratology studies most likely induces consecutive episodes of embryonic cardiac arrhythmia and embryonic death, thereby masking the teratogenic potential. This has been shown for cisapride (Skold *et al.*, 2002) and astemizole (Nilsson et al, in press). Malformations are only observed if the embryo survives the episode of hypoxia. Therefore, a study design with a single dose on a sensitive day of gestation allows detection of teratogenic potential of I_{Kr} blockers, as discussed in a recent review (Karlsson *et al.*, 2007).

In the clinical situation it is also difficult to identify teratogenic potential, even when human pregnancy outcome data are available. According to a recent guideline (EMEA, 2008) a negative study (no observed increase in malformations) with 300 drug exposed pregnancies and 300 appropriate controls only indicates that the risk for birth defects is not increased by more than 10 times. Even a thousand exposed pregnancies in such a study would only exclude a doubled risk. In the case of the pattern of malformations induced by phenytoin the situation may be even more complicated since each individual malformation (e.g. distal digital reductions, cleft lip or cardiovascular anomalies) may not be present in every case, increasing the risk of a pattern being overlooked. This suggests that there may be drugs used in pregnancy today that are teratogenic through an arrhythmia/hypoxia mechanism that are yet to be identified.

There may however be a way to approach this problem. Animal studies show that the malformations that are most commonly induced by selective I_{Kr} blockers are cardiovascular anomalies (Skold *et al.*, 2001). Also in a study designed to investigate the cogni-

tive effects of phenytoin, using concentrations chosen to not induce structural malformations, were cardiovascular anomalies observed (Vorhees, 1983). We can speculate that already at low concentrations the rhythm abnormalities are sufficient to induce cardiovascular anomalies, while more severe episodes of disrupted circulation and hypoxia are required for other abnormalities to manifest.

By applying this knowledge and combining it with information about I_{Kr} /hERG blocking potential, potentially human teratogenic drugs can be identified. This has already been suggested for the macrolide antibiotic erythromycin (Kallen *et al.*, 2005) and may also apply to tricyclic antidepressants such as clomipramine (Kallen & Otterblad Olausson, 2003) and certain SSRI antidepressants that also recently have been associated with cardiovascular teratogenicity in humans (Kallen & Otterblad Olausson, 2006; Merlob *et al.*, 2009).

Embryonic models are necessary to follow up observations like these and to test new substances for embryonic arrhythmogenic potential. The embryo ECG method presented in this thesis could provide such a model. With further development, the ECM cultured on MEA may also provide valuable information about embryonic cardiac sensitivity. Especially interesting is the prospect of a human embryonic cardiomyocyte model with cells derived from cardiac $Isl1^+$ progenitor cells.

9 CONCLUSIONS

- Phenytoin induces dose-dependent embryonic hypoxia during the susceptible stage for induction of malformations. The new digitalization method provides an objective analysis of the inter-beat interval and allows visualization of arrhythmias.

- The studied antiepileptic drugs induce concentration-dependent embryonic bradycardia and arrhythmia. At clinically relevant concentrations, combinations of antiepileptic drugs induce embryonic bradycardia.

- $Isl1^+$ cells are present in the human embryonic heart and have the capacity to differentiate into cardiomyocytes. Cardiospheres derived from embryonic hearts display rate dependence of the action potential duration and sensitivity to β-adrenergic stimulation.

- QT_C prolongation precedes drug-induced embryonic arrhythmia. The sensitivity and arrhythmias differ from the adult. Rat embryonic cardiomyocytes display a similar sensitivity as the embryonic heart but respond with different arrhythmias to drug exposure.

- I_K components are expressed and functional in the embryonic heart of human, rat and rabbit. The expression pattern suggests clinical relevance of the I_{Kr} -related teratogenic mechanism observed in animal studies.

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