**KCNJ11 knockout morula re-engineered by stem cell diploid aggregation**

Timothy J. Nelson, Almudena Martinez-Fernandez and Andre Terzic*

*Marriott Heart Disease Research Program, Division of Cardiovascular Diseases, Departments of Medicine, Molecular Pharmacology and Experimental Therapeutics, and Medical Genetics, Mayo Clinic, Rochester, MN 55905, USA*

KCNJ11-encoded Kir6.2 assembles with ATP-binding cassette sulphonylurea receptors to generate ATP-sensitive K⁺ (K\textsubscript{ATP}) channel complexes. Expressed in tissues with dynamic metabolic flux, these evolutionarily conserved yet structurally and functionally unique heteromultimers serve as high-fidelity rheostats that adjust membrane potential-dependent cell functions to match energetic demand. Genetic defects in channel subunits disrupt the cellular homeostatic response to environmental stress, compromising organ tolerance in the adult. As maladaptation characterizes malignant K\textsubscript{ATP} channelopathies, establishment of platforms to examine progression of K\textsubscript{ATP} channel-dependent adaptive behaviour is warranted. Chimeras provide a powerful tool to assay the contribution of genetic variance to stress intolerance during prenatal or post-natal development. Here, K\textsubscript{CNJ11} K\textsubscript{ATP} channel gene knockout ↔ wild-type chimeras were engineered through diploid aggregation. Integration of wild-type embryonic stem cells into zona pellucida-denuded morula derived from knockout embryos achieved varying degrees of incorporation of stress-tolerant tissue within the K\textsubscript{ATP} channel-deficient background. Despite the stress-vulnerable phenotype of the knockout, ex \textit{vivo} derived mosaic blastocysts tolerated intrauterine transfer and implantation, followed by full-term embryonic development in pseudopregnant surrogates to produce live chimeric offspring. The development of adult chimerism from the knockout ↔ wild-type mosaic embryo offers thereby a new paradigm to probe the ecogenetic control of the K\textsubscript{ATP} channel-dependent stress response. 

**Keywords:** ATP-sensitive K⁺ channel; chimerism; ecogenetics; genetic variance; Kir6.2; stress

1. **INTRODUCTION**

From conception to senescence, environmental challenges pose ongoing threats to organismal integrity (Seley 1955; McEwen 2007). Decoding of the continuous influx of stress signals is integral to the initiation and execution of the adaptive, cytoprotective response that secures stress tolerance and promotes evolutionary survival (Chien 1999; Degterev & Yuan 2008). Biosensors have been recognized as essential components in distress resolution, matching demand and ensuring safeguard of organ function (Barki-Harrington & Rockman 2003; Zingman et al. 2003). Failure to respond to stress load, in the context of a genetic defect and malfunction in sensor proteins, results in maladaptation and poor outcome underlying the centrality of ecogenetic homeostasis in disease avoidance and species preservation (Zingman et al. 2002a; Ashcroft 2007; Olson et al. 2007).

The ATP-sensitive K⁺ (K\textsubscript{ATP}) channel complex, a unique combination of an inward rectifier K⁺ channel and an ATP-binding cassette protein, is a prototypic metabolism-gated biosensor (Miki & Seino 2005; Nichols 2006; Zingman et al. 2007). K\textsubscript{ATP} channels operate as high-fidelity molecular rheostats adjusting membrane potential-dependent functions to match cellular energetic demands (Terzic et al. 1995; Alekseev et al. 2005). Underscoring the critical role for K\textsubscript{ATP} channels in coupling metabolic dynamics with electrical activity is the recognition that disruption of channel function is life threatening (Ashcroft 2005; Reyes et al. 2007). Dysfunction in K\textsubscript{ATP} channel gating has been linked to insulin secretory disorders, namely congenital hyperinsulinism and neonatal diabetes (Thomas et al. 1995; Dunne et al. 2004; Glynn et al. 2004; Babenko et al. 2006; Pearson et al. 2006; Ashcroft 2007; Lin et al. 2008). Beyond the isolated failure of pancreatic β-cells, mutations in KCNJ11, the gene encoding the pore-forming Kir6.2 subunit of K\textsubscript{ATP} Channels (Aguilar-Bryan et al. 1995; Inagaki et al. 1995), are pathogenic in a syndrome that encompasses diabetes, developmental delay and epilepsy (Proks et al. 2004; Hattersley & Ashcroft 2005; Glynn et al. 2006; Ashcroft 2007).

Kir6.2 is also integral to the make-up of myocardial K\textsubscript{ATP} channels (Inagaki et al. 1996), and targeted disruption of KCNJ11 generates Kir6.2-deficient mice that lack functional K\textsubscript{ATP} channels in ventricular myocytes (Suzuki et al. 2001). Intact Kir6.2 is required in cardiac adaptation to physiological and pathophysiological stress (Zingman et al. 2002a, 2003; Kane et al. 2006a; Tong et al. 2006; Yamada et al. 2006; Gumin et al. 2007). Moreover, K\textsubscript{ATP} channel malfunction has been implicated in the development...
and progression of heart disease (Hodgson et al. 2003; Kane et al. 2005). Originally discovered in cardiac myocytes (Noma 1983), K_ATP channels are abundant in the sarcosome where they assemble as heteromultimers of the Kir6.2 pore and SUR2A, the ATP-binding cassette regulatory sulphophylenurea receptor subunit (Inagaki et al. 1996; Lorenz & Terzic 1999; Nichols 2006; Bryan et al. 2007; Dupuis et al. 2008; Karger et al. 2008). Integrated with cellular metabolic pathways (Dzeja & Terzic 1998; Carrasco 2008), K_ATP channels contribute to cellular metabolic response during prenatal and post-natal development. Here, in a developmental platform based on diploid aggregation, we have established a mosaic chimeric offspring. Engineering knockout ES cells that produced chimeric embryos with wild-type embryonic stem cells. Following intrauterine surgical transfer, surrogate females support normal embryonic development and give birth to chimeric offspring.

2. MATERIAL AND METHODS

(a) Kir6.2 knockout

K_ATP channel knockout mice were generated by targeted disruption of the KCNJ11 gene that encodes the Kir6.2 channel pore, and backcrossed for five generations into a C57BL/6 background (Miki et al. 1998). Owing to proximity of the mutated KCNJ11 gene with the gene encoding for albino hair colour in the SV129 embryonic stem cells used to create the null mutant, Kir6.2 knockout mice remain white upon backbreeding into the black C57BL/6 line (Kane et al. 2004). Mice were kept under a 12 L : 12 D cycle and allowed free access to tap water and standard chow.

(b) Timed pregnancy of superovulating knockout donors

Female KCNJ11 gene knockout mice were treated with reproductive hormones to maximize the isolation of stage-specific embryos (Eakin & Hadjantonakis 2006). In brief, superovulation was achieved in three to four-week-old females at the final stage of pre-pubescent development. On day 1 at 14.00 h, female donors received a single intraperitoneal (i.p.) injection (5 units in 0.1 ml) of pregnant mare serum gonadotrophin (PMSG) using a 27-gauge needle (figure 1). Two days later at 13.00 h, donors received an i.p. injection (5 units in 0.1 ml) of human chorionic gonadotrophin (HCG). Knockout females were immediately paired with knockout studs to achieve timed mating that occurred

![Figure 1](http://rstb.royalsocietypublishing.org/)

Figure 1. Morula-stage embryos re-engineered through diploid aggregation. Flowchart of chimeric offspring derivation from random incorporation of embryonic stem cells into early stage embryos achieved through imposed diploid aggregation. Yellow box: Initial embryos are generated from timed pregnant KCNJ11 gene-deficient donors previously superovulated through hormonal activation using sequential i.p. injections of PMSG and HCG. Knockout embryos are harvested at 2.5 d.p.c. to collect at the morula stage. Zona pelucida is removed using acid Tyrode’s solution to prepare aggregation competent early embryos. Blue box: Simultaneously, embryonic stem cells are grown for two passages to produce low-density cultures that when digested are able to generate clumps of 8–15 pluripotent stem cells. Green box: Synchronized pseudopregnant surrogates are produced by appropriate selection of females in oestrus, and mated with vasectomized studs. Bottom: Mosaic morula generated after aggregation of KCNJ11 gene-deficient embryos with wild-type embryonic stem cells. Following intrauterine surgical transfer, surrogate females support normal embryonic development and give birth to chimeric offspring.
during the night on day 3 according to circadian rhythm dictated by the light/dark cycle. Superovulated females were removed from studs the following morning, and allowed to proceed through normal pregnancy. Knockout embryos, at 2.5 days post-coitum (d.p.c.), were harvested by retrograde flushing from the distal oviduct through the infundibulum using a 32-gauge needle. Superovulated donors produced up to 30 synchronized embryos in a single oviduct. 

(c) Collection of zona pelucida-denuded knockout morulae

Morula-stage embryos (figure 1) were washed in EmbryoMax M2 medium (Millipore, Billerica, MA) to remove cellular debris associated with oviduct flushing (Eakin & Hadjantonakis 2006). Glycoproteinaceous zona pellucida was removed to produce denuded morulae, competent for stem cell integration. A 35 mm culture dish was prepared with a drop of M2 and a drop of acid Tyrode’s solution at room temperature. Embryos, in groups of 20–30, were transferred with as little M2 medium as possible into the acid Tyrode solution, and continuously irrigated to keep neighbouring embryos separated until zona pelucida dissolved within 30–40 s. Once stripped of their zona pellucida, denuded morulae were washed in five drops of M2 followed by five drops of EmbryoMax KSOM (Millipore, Billerica, MA), preparing them for subsequent in vitro manipulation.

(d) Selection of wild-type embryonic stem cell clumps

Murine embryonic stem cells (R1-derived line) containing a single copy of the constitutively expressed β-galactosidase gene were maintained in Glasgow’s Minimum Essential Medium (BioWhittaker-Cambrex, Walkersville, MD) supplemented with pyruvate and l-glutamine (Cellgro, Mediatech, Inc. Herndon, VA), non-essential amino acids (Cellgro, Mediatech, Inc. Herndon, VA), β-mercaptoethanol (Sigma-Aldrich, St Louis, MO), 15 per cent foetal calf serum (PCS, Invitrogen Corporation, Carlsbad, CA) and leukaemia inhibitory factor (LIF; ESGRO, Chemicon International, Inc, Temecula, CA) and cocultured with inactivated mouse embryonic fibroblast feeders in a six-well plate on day 1 (Nelson et al. 2006, 2008). Embryonic stem cells were split 1/3, 1/6 and 1/12 on day 3 in order to ensure proper density for diploid aggregation (Nelson et al. 2004). On day 6, embryonic stem cells at approximately 60 per cent confluence were digested with 1 ml of trypsin for 4 min until the cells were loosely associated with each other. Gentle mechanical disruption was required to produce small clumps of the cells before adding 5 ml of growth medium to inactivate trypsin solution (Eakin & Hadjantonakis 2006). Care was taken to avoid producing single cell suspensions. The mixture was pre-plated on tissue culture plates to allow feeder cells to attach before collection of embryonic stem cell clumps. Selected clumps were washed in five drops of M2 medium followed by five drops of KSOM medium for subsequent diploid aggregation (figure 1).

(e) Synchronized pseudopregnancy of surrogate females

Surrogate mothers are required for proper in vivo development of embryos re-engineered outside of the natural environment. CD-1 females, at least six to eight weeks old, were maintained in a colony of 50–70 animals (Eakin & Hadjantonakis 2006). On day 4, females in oestrus were identified by careful examination of vaginal changes indicated...
3. RESULTS

(a) *KCNJ11* gene-deficient morulae tolerate ex utero manipulation

Chimera blastocytes resulting from integration of embryonic stem cells with morula have provided a powerful tool to study developmental biology from early embryonic stages to adult phenotypes (Wood *et al.* 1993; Tam & Rossant 2003). Compact morulae were here harvested from *KCNJ11* gene knockout donors after undergoing superovulation and mating with knockout studs. The optimal age of female donors was three weeks to maximize the number of appropriate morula-stage embryos with 20 donors producing 200–300 viable embryos for each experiment. Traditionally, the wild-type CD-1 mouse strain is used as a donor at this age due to robust capacity to produce large number of embryos following a super-ovulation generating 30–40 high-quality embryos in a single uterus. The efficiency of morula production in *KCNJ11* gene knockout donors was significantly less, with the average uterus producing 5–15 appropriate embryos, despite optimization of the protocol. This was in part due to a high degree of unfertilized single cells and atypical eight-cell embryos in *KCNJ11* gene knockout donors. Early morula-stage embryos were collected, washed and processed through acid Tyrode’s solution to remove the zona pellucida (figure 2a and 2b). Again, compared with traditional wild-type CD-1 embryos, recovery of *KCNJ11* gene knockout embryos after *in vitro* removal of zona pellucida was less efficient. The zona denudation with acid washing resulted in the destruction of approximately a third of knockout embryos compared with less than 5 per cent of CD-1 embryos. A significant percentage of knockout embryos were destroyed as individual cells of the morula were completely dissociated from each other following acid wash, which eliminated the structure of the embryo required for aggregation and normal development. Morulae that tolerated the stress of denudation were selected for subsequent embryonic stem cell aggregation. Embryonic stem cells labelled with β-galactosidase gene allowed lacZ staining to detect wild-type embryonic stem cell-derived progeny at subsequent stages of development. An embryonic stem cell clump of 8–15 cells was collected upon careful enzymatic digestion (figure 2c), and placed into a well containing two aggregation competent knockout donors. Early morula-stage embryos were collected, washed and processed through acid Tyrode’s solution to remove the zona pellucida (figure 2d). The complementation assay allowed integration of embryonic stem cells into *KCNJ11* gene knockout embryos at the early morula stage. The efficiency of blastocyte formation from knockout morula re-engineered with wild-type embryonic stem cell aggregation was also decreased compared with CD-1 counterparts, and moreover the knockout morula required longer observation for an additional approximately 4–6 hours to achieve full maturation of blastocyte cavitation. Thus, *KCNJ11* gene-deficient embryos were vulnerable to stressful in vitro manipulation; however, a sufficient number of *KCNJ11* gene-deficient morulae were able to incorporate embryonic stem cells and advance beyond the morula stage.

(b) Diploid aggregation creates mosaic knockout ↔ wild-type embryos

Embryonic stem cell integration into developing embryonic tissue through diploid aggregation is typically achieved using wild-type donor embryos aggregated with a variety of mutant embryonic stem cells (Eakin & Hadjantonakis 2006). Despite the lower overall efficiency...
of $\text{KCNJ11}$ gene knockout embryo production, aggregation competent progeny enabled the generation of mosaic embryos using wild-type embryonic stem cells labelled with a constitutively expressed β-galactosidase (lacZ) gene from the elongation promoter (Nelson et al. 2006). Blastocyes resulting from the aggregation between knockout donors and wild-type embryonic stem cells were collected from in vitro culturing media on day 7 of the procedure. Normal morphology with proper cavitation and inner cell mass formation was observed (figure 3(ii)). These engineered early stage embryos were indistinguishable from traditional wild-type CD-1 embryos at the 3.5 d.p.c. developmental stage. Re-engineered embryos were stained for lacZ expression and demonstrated robust expression of wild-type embryonic stem cell progeny in the majority of embryos at day 7 (figure 3(iii)). Thus, engraftment of wild-type embryonic stem cells was maintained in $\text{KCNJ11}$ gene knockout embryos during in vitro blastocyte formation and provided the opportunity to examine chimeric embryo formation between Kir6.2-deficient embryos and wild-type embryonic stem cells. Upon intrauterine transplantation and proper development, mosaic blastocyes differentiated into morphologically normal, age-appropriate embryos at 9.5 d.p.c. (figure 3b(i)). Staining for wild-type embryonic stem cell-derived tissue expressing lacZ demonstrated embryonic stem cell incorporation throughout embryonic tissues including the heart, brain, somites, pharyngeal arches and primordial liver (figure 3b(iii)). Chimeric $\text{KCNJ11}$ gene knockout embryos incorporated wild-type embryonic stem cells during early stages of embryonic organogenesis, demonstrating functional chimerism in the embryo and justifying the experimental approach to generate adult chimeras.

(c) Assortment of knockout and wild-type tissues in viable adult chimera

Surrogate mothers of the CD-1 background were used to support proper in utero development for chimeric embryos (figure 3). High-throughput diploid aggregation was required to produce sufficient numbers of chimeric blastocyes due to the stress intolerance of the $\text{KCNJ11}$ gene-deficient background. Further vulnerability of this background mouse strain was realized when the majority of born pups were unexpectedly destroyed by CD-1 surrogate mothers within 3 postnatal days. This suggests a selection process by the surrogate mothers to identify unfit offspring and eliminate pups according to perceived maladaptive behaviours, which is less common when chimeras are derived from the traditional CD-1 background strain. The white coat colour of the $\text{KCNJ11}$ gene-deficient background allowed chimeric animals to be identified based on dark hair colour derived from embryonic stem cell contribution. Of note, three-week-old pups derived from the $\text{KCNJ11}$ gene-deficient background using the diploid aggregation platform produced offspring with varying degrees of chimeric coat colours, indicating a spectrum of wild-type embryonic stem cell incorporation. Diploid aggregation-derived chimeric pups were larger than non-chimeric littersmates, suggesting a disparity between the two cohorts (figure 4). The overall efficiency of adult chimea production using $\text{KCNJ11}$ gene-deficient morula was less than 0.01 per cent initially, and has been increased to more than 1 per cent after optimization to minimize unnecessary stress induced at each stage of the production process. Despite the low number of viable offspring using the $\text{KCNJ11}$ gene-deficient donors, the high percentage of chimeric offspring according to coat colour identification may suggest an overall survival advantage conferred by wild-type embryonic stem cell-derived tissues during prenatal and perinatal development of the $\text{KCNJ11}$ gene-deficient background.

4. DISCUSSION

An emerging body of evidence implicates, in the adult, the $\text{K}_{\text{ATP}}$ channel as a unifying molecular coordinator of metabolic well-being under stress, ensuring energetic homeostasis in health and disease (Zingman et al. 2003; Nichols 2006; Ashcroft 2007). Less is, however, known regarding the contribution of $\text{K}_{\text{ATP}}$ channel-dependent adaptation during development. Yet the activity of ion channels and pumps, and their molecular regulators, is an increasingly recognized contributor to embryonic development as initial mapping unravels complex functional roles in non-mammalian phylogeny (Cheng et al. 2002; Akasaka et al. 2006). In mammalian systems, a traditional approach to probe the genetic control of prenatal and post-natal development has included engineering chimeric constructs (Eakin & Hadjantonakis 2006). Here, we provide the first $\text{K}_{\text{ATP}}$ channel knockout ↔ wild-type chimera as a platform to monitor the outcome of gene–environment interactions from conception to senescence.

Specifically, in the $\text{KCNJ11}$ null mutant background, diploid aggregation with wild-type embryonic stem cells added wild-type blastomeres to the $\text{K}_{\text{ATP}}$ channel knockout morula yielding a mosaic blastocyst. Diploid aggregation allows the integration, in the context of the host morula, of an independent source of pluripotent progenitors which is in principle equally competent to mature into all lineages (Wood et al. 1993). Although genetic modifications have been typically studied in the milieu of wild-type background providing the foundation for developmental biology and lineage allocation (Tam & Rossant 2003), diploid aggregation using a mutant host—as developed herein—allows the study of putative disparity between $\text{K}_{\text{ATP}}$ channel knockout versus wild-type blastomeres in a cell-autonomous (environment-independent) as well as non-cell-autonomous (environment-dependent) paradigm. In this way, the study of embryonic ecogenetics is enabled through direct manipulation of either the progenitor cell or its environment.

The $\text{K}_{\text{ATP}}$ channel knockout morula displayed a fragile phenotype on removal of the zona pellucida as demonstrated by frequent dispersion of blastomeres and ensuing embryo destruction, in contrast to the wild-type counterparts, which maintained functional and structural integrity under equivalent stress load. While stress intolerance associated with $\text{K}_{\text{ATP}}$ channel-deficiency is well documented in the adults (Kane et al. 2005; Miki & Seino 2005; Nichols 2006; Ashcroft 2007), the vulnerability observed here at the morula stage indicates that ablation of the Kir6.2 channel pore
causes disruption of the K<sub>ATP</sub> channel-dependent cytoprotection early in development. Such inherent maladaptation to stress is in line with primordial K<sub>ATP</sub> channel-dependent functions in the regulation of proliferation, cell cycle and cell migration (Cheng et al. 2002). In the context of the stress-vulnerable K<sub>ATP</sub> channel knockout morula, aggregation-derived knockout wild-type mosaic blastocysts tolerated intrauterine transfer and implantation, followed by full-term embryonic development in pseudopregnant surrogates to produce live chimeric offspring. Longitudinal analysis of chimerism throughout pre- and post-natal development thereby allows the dissection of K<sub>ATP</sub> channel-mediated adaptive behaviour for each developing tissue and organ beyond the cell source in the antecedent morula.

In summary, this study re-engineers the K<sub>ATP</sub> channel knockout morula into a knockout ↔ wild-type chimera offering a new technological platform to probe the disparity between K<sub>ATP</sub> channel-dependent and independent pathways underlying the adaptive stress response. Chimerism generated through the competing fitness in the adaptive response of Kir6.2-rich versus Kir6.2-depleted progenitors opens a unique window to deconvolute the process of evolutionary selection, according to K<sub>ATP</sub> channel functionality.

Figure 3. Mosaic blastocyes derived from labelled embryonic stem cells differentiate as components of multiple organs. (a) 3.5 d.p.c. Mosaic blastocyes: (i) (WT ↔ WT) normal morphology of blastocyte stage embryos with proper cavitation and inner cell mass formation was observed with (ii) (KO ↔ WT) lacZ stain highlighting the presence of wild-type embryonic stem cell progeny in the majority of late stage morula: or blastocyes. (b) 9.5 d.p.c. Chimeric blastocyes (i) (WT ↔ WT) upon intrauterine transplantation and proper development, mosaic blastocyes differentiated into morphologically normal 9.5 d.p.c. with (ii) (KO ↔ WT) lacZ stain revealing wild-type embryonic stem cell-derived tissue. Expression of lacZ demonstrated wild-type embryonic stem cell-derived progeny throughout the embryo in tissues such as the heart (H), brain (B), somites (S), pharyngeal arches (P) and primordial liver (L).

Figure 4. Chimeric animals produced from KCNJ11-deficient embryos and wild-type embryonic stem cells. Surrogate mothers support normal development and give birth to live (three-week-old) chimeric offspring. (a) KCNJ11 knockout wild-type chimera animals develop combination of white and dark coat colours. (b) Three-week-old litter mates: chimera animals are larger than non-chimera animals.

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