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Deficiency in the mouse mitochondrial adenine nucleotide translocator isoform 2 gene is associated with cardiac noncompaction☆

Jason E. Kokoszka [a], Katrina G. Waymire [b], Adrian Flierl [c], Katelyn M. Sweeney [d], Alessia Angelin [d], Grant R. MacGregor [b], Douglas C. Wallace [d,]*

[a] Forensic Biology Section, Alabama Department of Forensic Sciences, Annex C, Mobile, AL 36617, United States
[b] Department of Developmental and Cell Biology, University of California Irvine, Irvine, CA 92697-2300, United States
[c] The Parkinson's Institute, Sunnyvale, CA 94085, United States
[d] Center for Mitochondrial and Epigenomic Medicine, Children's Hospital of Philadelphia, Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA 19104, United States

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ABSTRACT

The mouse fetal and adult hearts express two adenine nucleotide translocator (ANT) isoform genes. The predominant isoform is the heart–muscle–brain ANT-isoform gene 1 (*Ant1*) while the other is the systemic *Ant2* gene. Genetic inactivation of the *Ant1* gene does not impair fetal development but results in hypertrophic cardiomyopathy in postnatal mice. Using a knockin X-linked *Ant2* allele in which exons 3 and 4 are flanked by *loxP* sites combined in males with a *protamine 1* promoter driven Cre recombinase we created females heterozygous for a null *Ant2* allele. Crossing the heterozygous females with the $Ant2^{fl}$, *PrmCre*(+) males resulted in male and female ANT2-null embryos. These fetuses proved to be embryonic lethal by day E14.5 in association with cardiac developmental failure, immature cardiomyocytes having swollen mitochondria, cardiomyocyte hyperproliferation, and cardiac failure due to hypertrabeculation/noncompaction. ANTs have two main functions, mitochondrial–cytosol ATP/ADP exchange and modulation of the mitochondrial permeability transition pore (mtPTP). Previous studies imply that ANT2 biases the mtPTP toward closed while ANT1 biases the mtPTP toward open. It has been reported that immature cardiomyocytes have a constitutively opened mtPTP, the closure of which signals the maturation of cardiomyocytes. Therefore, we hypothesize that the developmental toxicity of the *Ant2* null mutation may be the result of biasing the cardiomyocyte mtPTP to remain open thus impairing cardiomyocyte maturation and resulting in cardiomyocyte hyperproliferation and failure of trabecular maturation. This article is part of a Special Issue entitled 'EBEC 2016: 19th European Bioenergetics Conference, Riva del Garda, Italy, July 2–6, 2016', edited by Prof. Paolo Bernardi.

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1. Introduction

Mitochondrial dysfunction has frequently been associated with cardiomyopathy. In adults mitochondrial dysfunction can result in hypertrophic or dilated cardiomyopathy [1–3]. In pediatrics, between 0.4 and 5% of live birth infants present with a cardiac malformation. Common among these congenital malformations are ventricular septal defects with cardiac noncompaction being a relatively rare but severe congenital cardiac abnormality [4–6].

Noncompaction of the ventricular myocardium is the persistence of multiple prominent ventricular trabeculations and deep inter-trabecular recesses. Noncompaction is thought to represent an arrest of endomyocardial morphogenesis, which normally occurs between weeks 5 and 8 of human fetal life. Proliferation of the embryonic cardiomyocytes creates the trabecular network which evolves through the gradual compaction of the myocardium, transformation of large intratrabecular spaces into capillaries, and the evolution of the coronary circulation, a process that typically progresses from the epicardium to the endocardium and from the base of the heart to the apex [6].

Mitochondrial dysfunction and disruption of mitochondrial dynamics and the intrinsic pathway for apoptosis have been associated with a variety of cardiac pathologies [1,7]. In a number of cases ventricular noncompaction has been associated with alterations in nuclear DNA (nDNA) coded mitochondrial genes. Barth Syndrome patients and their mouse models can present with noncompaction caused by mutations in the X-linked Tafazzin gene which perturbs mitochondrial cardiolipin metabolism [8,9]. Mice lacking cytochrome c die by E10.5 [10]. Mice with defects in the *Mitofusion 1* & *2* genes or lacking the apoptosis associated genes for caspases 3, 7, 8, FADD and c-FLIP can developed cardiac

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* Corresponding author at: Center for Mitochondrial and Epigenomic Medicine (CMEM), Children's Hospital of Philadelphia, Department of Pathology and Laboratory Medicine, University of Pennsylvania, Colket Translational Research Building, room 6060, 3501 Civic Center Boulevard, Philadelphia, PA 19104, United States.

E-mail address: wallaced1@email.chop.edu (D.C. Wallace).

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noncompaction [11–13]. Mice with defects in cardiac Parkin–PINK1-modulated mitophagy can manifest perinatal lethality due to impaired replacement of fetal mitochondria with adult mitochondria [14,15]. Novel mitochondrial DNA (mtDNA) sequence variants have also been reported in noncompaction patients, both in DNAs extracted from patient blood samples [16] and from explanted hearts [17]. Noncompaction hearts have also been found to have a reduced mtDNA copy number in association with mitochondrial ultrastructural abnormalities [17].

To further define the role of mitochondrial dysfunction in cardiomyopathy, we have systemically inactivated the mouse *Ant1* and *Ant2* genes. The ANTs mediate the exchange of ATP and ADP across the mitochondrial inner membrane and thus are central to mitochondrial energy production. They also modulate the mtPTP and thus regulate the intrinsic pathway of apoptosis.

The nature of ANT regulation of both energy metabolism and apoptosis is complex, due to there being multiple ANT isoforms with slightly different properties. Humans have four ANT isoforms [18,19]: ANT1 which is predominantly expressed in the heart and muscle [20–24], ANT2 which is a systemic and inducible [25–27], ANT3 which is also systemic [26], and ANT4 which is testis specific [28]. Mice have three isoforms [29–31]: ANT1 which is expressed in the heart–muscle–brain [30]; ANT2 which is X-linked, expressed in most tissues, and inducible [31]; and ANT4 which is predominantly testis specific [32]. Human ANT1 and ANT3 have been proposed to favor export of ATP from the mitochondrial matrix into the cytosol while ANT2 has been proposed to be kinetically capable of importing cytosolic ATP into the mitochondrial matrix under hypoxic conditions and in cancer cells [33–37].

In addition to the transport of ADP and ATP across the mitochondrial inner membrane, the ANTs have been found to regulate the mtPTP [38]. The mtPTP spans the mitochondrial inner membrane and current data indicates that the mtPTP is composed of components of the ATP synthase, either ATP synthase dimers or the C-ring [39–43]. In differentiated cells, a variety of factors can initiate opening of the mtPTP and apoptosis including reduced mitochondrial inner membrane potential, elevated ADP, elevated reactive oxygen species (ROS), Ca^{++} overload, diamide, and atractyloside, a ligand of the ANTs [2,38].

The different ANT isoforms have different effects on the mtPTP. Over-expression of human ANT1 and ANT3 by transformation of established cell lines with extra *ANT1* and *ANT3* gene copies has been shown to induce apoptosis. However, over-expression of *ANT2* does not have this effect [37,44–47]. The induction of apoptosis by ANT1 does not rely on ATP/ADP transport, but rather is related to amino acids 102–141, the region of greatest divergence between the ANT isoforms [44]. This suggests that the pro-apoptotic action of ANT1 over-expression most likely involves protein–protein interaction. ANT1 but not ANT2 is associated with the IκBα–NFκB complex which is sequestered within the mitochondrion intermembrane space. Over-expression of ANT1 then traps the IκBα–NFκB complexes in the mitochondrion impeding its migration to the nucleus. In the nucleus NFκB transcriptionally activates the *Bcl-XL*, *c-IAP2*, and *Sod2* (MnSOD) genes [46,47]. *Bcl-XL* and *c-IAP2* are anti-apoptotic polypeptides and MnSOD is a mitochondrial matrix enzyme that converts superoxide anion to hydrogen peroxide. Co-expression of cyclophilin D or NFκB with ANT1 inhibits apoptosis confirming that over-expression of ANT1 limits the levels of these anti-apoptotic factors [44,46]. Over-expression of ANT1 must then reduce *Bcl-XL*, *c-IAP2*, and *Sod2* expression favoring the activation (opening) of the mtPTP and increasing matrix ROS production which in differentiated cells would initiate the intrinsic pathway of apoptosis.

Mouse liver mitochondria, which only contain ANT2, still retain a tBHP-sensitive mtPTP when *Ant2* is inactivated. However, the ANT2-deficient mtPTP becomes insensitive to atractyloside and the mtPTP of the ANT2-deficient hepatocytes is intrinsically more sensitive to activation and opening when the cells are treated with increasing concentrations of the calcium ionophore Br-A23187 [38]. These observations suggest that increased ANT1 expression favors the opening of the mtPTP while increased ANT2 expression favors mtPTP closure.

Inactivation of the *Ant1* gene in the mouse has no apparent negative effect on development, with *Ant1* null mice being born at a similar frequency as *Ant1* positive mice. However, by three months of age, the *Ant1* null mice develop a hypertrophic cardiomyopathy [30] which can progress to dilated cardiomyopathy over their two year life span [48]. In humans inactivating mutations in the *ANT1* gene also permit normal development, but results in lifelong cardiomyopathy, the severity of which can be modulated by the individual's mtDNA lineage [49–51]. The systemic inactivation of the mouse *Ant1* gene results in partial inhibition of cardiac mitochondrial ADP-stimulated respiration and increased mitochondrial ROS production. However, the severity of these cardiac effects is not as marked as for the skeletal muscle since in the heart, both ANT1 and ANT2 are expressed while in the muscle ANT1 is the only observed isoform [30,31,52].

Given the cardiac effects of *Ant1* inactivation and the co-expression of ANT1 and ANT2 in the heart, it became important to determine the effect of inactivating the *Ant2* gene on heart function. Surprisingly, unlike *Ant1* null mice, *Ant2* null mice are embryonic lethal. This is associated with a striking cardiac developmental defect including ventricular hypertrabeculation/noncompaction with swollen cardiomyocyte mitochondria, cardiomyocyte hyperproliferation, embryonic lethality by E14.5, and in rare cases neonates with congenital heart defects. Hence, certain types of mitochondrial dysfunction can also contribute to congenital cardiomyopathy.

2. Results

2.1. Differential expression of Ant1 and Ant2 in adult mouse tissues

Mouse somatic tissues differentially express *Ant1* and *Ant2* (Fig. 1). *Ant1* mRNA is present at relatively high levels in the adult skeletal muscle, heart, and to a lesser extent brain and kidney but is absent in the liver. Similarly, ANT1 protein is present at high levels in the heart, skeletal muscle, brain, and to a lesser extent kidney, but is undetectable in the liver. *Ant2* mRNA is present at high levels in the kidney, brain, liver, and heart but is at very low levels in the skeletal muscle, and ANT2 protein is present at high levels in the heart, liver, kidney and brain but is virtually undetectable in the adult skeletal muscle (Fig. 1). Hence, both *Ant1* and *Ant2* are expressed in the adult heart.

2.2. Genetic analysis of Ant2-null mice

To determine the impact of inactivating the *Ant2* gene on cardiac development we employed our mice harboring a conditional knockout allele of the *Ant2* gene [38]. *Ant2* is X-linked [29] and we have substituted the native allele for one in which exons 3 and 4 were flanked by *loxP* sites in the 5′-intron and 3′-untranslated region, creating a functional *Ant2* “floxed” allele ($Ant2^{fl}$, or X^{fl}) [38].

In the current studies, we generated animals lacking ANT2 in all tissues (i.e. a global knockout of the *Ant2* gene) using a *Cre* recombinase transgene transcribed from the *protamine* 1 promoter [53] ($X^{fl}Y$ *PrmCre*(+)). Since the *protamine* promoter is only active during spermiogenesis and genetically haploid sperm share gene products via inter-cellular bridges [54] males hemizygous for both *PrmCre*(+) and the X^{fl} chromosome should transmit a null allele of *Ant2* (X^{del}) in which the last two exons are deleted.

$X^{fl}Y$, *PrmCre*(+) males were mated with females heterozygous for the X^{fl} and wild type (X^{+}) alleles, resulting in female progeny receiving one deleted allele from the father ($X^{del}X^{fl}$ or $X^{del}X^{+}$) while the males receive either the X^{fl} or X^{+} from the mothers plus the Y from the fathers. Thus all mice have one active *Ant2* gene. The average litter size of these matings was 10.69 ± 0.73, similar to that seen in crosses in which the male transmitted a functional allele (10.80 ± 1.24, P value = 0.93).

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