Fetal Heart Rate Predictors of Long QT Syndrome

Running title: Mitchell et al.; Fetal Heart Rate Predictors of LQTS

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Journal Subject Codes: [5] Arrhythmias, clinical electrophysiology, drugs; [109] Clinical genetics; [41] Pediatric and congenital heart disease, including cardiovascular surgery
Abstract:

Background—Fetal long QT syndrome (LQTS) is associated with complex arrhythmias including torsades de pointes and 2° atrioventricular (AV) block. Sinus bradycardia has also been associated with fetal LQTS, but little is known of this rhythm manifestation. Our purpose was to characterize the fetal heart rate (FHR)/gestational age (GA) profile of fetal LQTS.

Methods and Results—We ascertained fetal LQTS subjects by family history (Group 1) or fetal arrhythmia referral (Group 2). We compared FHR in LQTS subjects vs. normal fetuses. To identify FHR predictors of LQTS, we calculated a “bradycardia index” as % of LQTS FHR recordings either ≤ 110 bpm (obstetrical standard) or ≤ 3rd percentile for GA.

Among 42 LQTS subjects, 26 were in Group 1 and 16 in Group 2. There were 536 normal fetuses. The bradycardia index was only 15% for FHR≤110 bpm, but 66% for FHR ≤ 3rd percentile for GA. Ten fetuses with complex arrhythmias also had severe and sustained sinus bradycardia throughout gestation. Identifying a fetal proband in Group 2 resulted in LQTS diagnosis in 9 unsuspected members of 6 families.

Conclusions—FHR varies by GA in both normal and LQTS fetuses. Postnatal evaluation of neonates with FHR ≤3rd percentile for GA may improve ascertainment of LQTS in fetuses, neonates and undiagnosed family members.

Key words: arrhythmia; fetal cardiovascular abnormalities; long QT syndrome
Introduction

Long QT syndrome (LQTS) is reported to have an incidence of 1 in 2500 individuals. While QT interval prolongation may be an incidental finding, LQTS typically presents in adolescence or young adult life with syncope, sudden death or cardiac arrest \(^1,^2\). Less frequently, LQTS presents in the perinatal (fetal/neonatal) period; in this setting morbidity and mortality are high, and torsades de pointes (TdP) and \(^2^\circ\) atrioventricular (AV) block are signature rhythms \(^3^,^6\). Sinus bradycardia is also a manifestation of fetal LQTS and is reported to be more common than TdP and \(^2^\circ\) AV block. For example, as many as 44-66\% of fetuses diagnosed with LQTS presented with sinus bradycardia at 26-40 weeks of gestation \(^3^,^6^,^8\). In most reports, a fetal heart rate (FHR) \(\leq 110\) bpm at any gestational age (GA) raised suspicion of LQTS. Indeed, FHR of \(\leq 110\) bpm at any GA is the obstetrical definition of sinus bradycardia \(^9\). However, little is known of the sensitivity of this finding and how it relates to the subsequent diagnosis of LQTS.

It is well known that FHR in the normal fetus decreases during gestation from about 175 bpm at 10 weeks to 138 bpm at 40 weeks. This phenomenon is believed to be due to the increasing dominance of the parasympathetic nervous system on heart rate control as gestation progresses \(^10,^11\). Despite the association between fetal bradycardia and LQTS, the FHR/GA profile, or the range of FHRs of subjects with LQTS, has not been defined. We wondered if there might be a pathologic FHR in the setting of 1:1 AV conduction that was below normal for GA but \(> 110\) bpm that might be a sensitive marker for fetal LQTS. We hypothesized that the FHR/GA profile of LQTS individuals would be different than that of normal individuals and there might be GA-specific or genotype-specific FHR predictors of LQTS.

The purposes of this study were first, to define the FHR/GA and rhythm profile of individuals with LQTS mutations and compare this profile with that of a normal control group.
Second, we hoped to develop FHR criteria that would improve the recognition of LQTS in the perinatal period.

**Methods**

This was a study of fetal cardiac rhythm in pediatric subjects with a clinical and genetic diagnosis of LQTS. Participants were recruited at 3 medical centers. (¹ Advocate Hope and Lutheran General Children’s Hospitals, Chicago IL; University of Tsukuba, Tsukuba, Ibaraki, Japan; and University of Utah and Primary Children’s Medical Center, Salt Lake City, UT.)

Fetal heart rate in LQTS subjects across GA were compared to FHR from a normal control group across similar GA. Approval from the institutional review boards of each participating center was obtained.

**Study groups**

**Recruitment of LQTS subjects**

To avoid possible ascertainment bias, we divided LQTS subjects into two groups. Subjects in Group 1 had a family history of genetically confirmed LQTS and were under increased surveillance because of a risk of LQTS recurrence. Group 2 consisted of fetuses referred for evaluation of cardiac arrhythmia; in some cases Group 1 subjects were siblings of individuals in Group 2.

**Recruitment of normal subjects**

Normal subjects were recruited from Advocate Christ Medical Center and Hope Children’s Hospital. Inclusion criteria were a normal obstetrical ultrasound or a normal fetal echocardiogram. Indications for obstetrical ultrasound of the normal subjects at 7-15 weeks were viability and nuchal translucency screening; indications for echocardiography of the normal
subjects at 16-40 weeks were maternal diabetes, advanced maternal age, medication exposure or suspicion of fetal disease, e.g. family history of a congenital heart defect. Exclusion criteria for normal subjects at 7-40 weeks GA included abnormal nuchal translucency measurement, fetal cardiac arrhythmia or history of arrhythmia, congenital heart defect, or clinically significant noncardiac malformation, e.g. spina bifida, or known chromosome abnormality. The FHR of 12 infants evaluated for a maternal or paternal history of LQTS but with negative genetic testing were included in the normal subjects. No subject in the normal Group was related to any subject in LQTS Groups 1 or 2.

**Fetal heart rate measurements**

**LQTS subjects**

FHR of the LQTS subjects were obtained from obstetrical records throughout the mother’s pregnancy. They were derived from M-mode measurements of ventricular or atrial contractions from 5 consecutive cardiac cycles when the fetus was still, and/or by Doppler auscultation of the FHR routinely performed at monthly or twice monthly visits to the obstetrical care provider. For fetuses with AV block, FHR was determined either during intermittent sinus rhythm or by measuring the atrial rate. Rhythms of LQTS subjects were classified as either sinus or complex, specifically TdP and/or 2° AV block.

**Normal subjects**

Method for determination of FHR in normal fetuses was GA dependent. FHR at 7-15 weeks of gestation were measured from atrial or ventricular M-mode waveforms of 5 consecutive cardiac cycles obtained during routine obstetrical ultrasounds. FHR at 16-40 weeks of gestation were measured from the aortic or pulmonary valve Doppler waveforms of 5 consecutive cardiac cycles obtained during fetal quiescence. Data were obtained from 10 fetuses for each week of
gestation from 7-40 weeks.

**LQTS diagnosis**

The diagnosis of LQTS was based on findings of a positive genetic test for LQTS. All genetic testing was performed in commercial genetic testing laboratories. Samples were tested for either 12 (GeneDx, Gaithersburg, MD) or 13 (Familion, Transgenomic Inc., New Haven, CT) LQTS gene subtypes. Only genetic variants reported to be deleterious were considered to be mutations; variants reported to be of uncertain significance were not considered pathologic. Mutations were classified as LQTS gene type, compound (more than one deleterious mutation), uncharacterized (no mutation in a known LQT gene) or untested. The presence of a signature LQTS rhythm, TdP or 2° AV block, in the fetal or neonatal period was considered confirmatory of the LQTS diagnosis, even if genetic testing revealed no mutation in a known LQTS gene.

The QT interval on a postnatal 12 lead ECG was corrected (QTc) by both Bazett’s and Frederica’s formulas and reported for LQTS subjects. Subjects in Group 1 had an ECG at the time of their initial evaluation during infancy or childhood, while those in Group 2 had an ECG during the first 24 hours of life.

**Fetal heart rate analysis**

We determined the 3rd, 50th and 97th percentiles for FHR/GA of the normal subjects by logarithmic regression analysis. For the purposes of this study, we defined bradycardia in 2 different ways: either independent of GA (FHR ≤ 110 bpm, the obstetrical definition of bradycardia) or dependent upon GA (FHR ≤ 3rd percentile for GA).

**Statistical analysis**

The FHR (mean ± standard error) was calculated for normal subjects and LQTS subjects. To maximize statistical power, we grouped the FHR data into 3 categorical GA groups: 1) < 21
weeks, 2) 21-30 weeks, and 3) 31-40 weeks and compared normal and LQTS FHR in the three
GA groups. A mixed effect model was performed taken the dependency of patients within the
same family into account. In order to eliminate maternal beta adrenergic blockade therapy as a
confounding variable for observed FHR differences between normal and LQTS subjects, we
compared FHR of treated and untreated mothers with LQTS by Mann-Whitney non-parametric
testing. A two-tailed $P$-level of < 0.05 was considered statistically significant. All analyses were

**Bradycardia index of LQTS subjects**

Once we derived the 3rd, 50th and 97th percentiles for the normal subjects at each GA, we
calculated a bradycardia index for each LQTS fetus using both definitions of bradycardia. In
other words, the bradycardia index was the ratio of FHR measures that were either $\leq 110$ bpm or
$\leq 3rd$ percentile for GA compared to the total number of FHR measures for that fetus. Because
the number of subjects in each genotype group was small, we did not seek to define a genotype-
specific effect on FHR or bradycardia index within categorical age groups or between Groups 1
and 2.

**Results**

**LQTS subjects**

The descriptions of the LQTS cohorts in Group 1 (referred with a family history of LQTS)
(n=26) and Group 2 (referred for arrhythmia evaluation) (n=16) are summarized in Table 1.
Among the 42 subjects, a diagnosis of LQTS was made during fetal or neonatal life in 32
subjects; in 10 subjects in Group 1, the diagnosis was made later during infancy or childhood.
The QTc intervals corrected by Bazett’s formula ranged from 450-700 (mean 582) ms and
corrected by Frederica’s formula ranged from 394-660 (mean 471) ms. Bazett’s correction resulted in a prolonged QTc (≥ 450 ms) in 95% of genetically proven LQTS subjects, while use of Friderica’s correction identifies only 59 % of genetically proven LQTS subjects as having a prolonged QTc. The corrected QT intervals by both formulae are shown in the Supplemental Table. Several members of Group 1 were in previously reported families (12-14). Siblings of 4 families were included in Group 1: subjects #10, #11 and #12; subjects #17 and #18, subjects #19 and #20, and subjects #21 and #22. Subjects #41 and #42 in Group 2 were twins. At the time of initial assessment, no Group 2 subject was known to have affected family members; however, subsequent diagnosis in the fetal proband led to a genetic diagnosis of LQTS in undiagnosed members of 6/16 (38%) families. These family members (subjects #5, #6, and #7) were included in Group 1 after diagnosis of LQTS in subjects #32, #33 and #37.

The mean GA at delivery was slightly less for Group 2 (36.4 ± 2.8 weeks) compared to Group 1 (38.0 ± 2.7 weeks) subjects, but this difference was not significant (P = 0.08). The mean GA of referral for subjects in Group 2 was 27.6 ± 4.5 weeks. Five subjects in Group 2 were delivered prematurely (≤ 35 weeks of gestation) because of uncontrolled arrhythmia or fetal distress; one fetus died in utero from uncontrolled arrhythmia and severe hydrops (Subject # 40).

Among mothers with LQTS, 13 were treated with beta adrenergic blocking agents during pregnancy: 11 throughout pregnancy and 2 during 3rd trimester only. The FHR was not different in fetuses whose mothers were treated (130.1 ± 8.2 bpm) or untreated (127.5 ± 13.6 bpm) (P = 0.6).

**LQTS mutations in group 1 vs. group 2**

Mutation in a known LQT gene was found in most subjects (92%) who underwent genetic testing (95%): 23 with LQT1, 4 with LQT2, 6 with LQT3, 2 with LQT5 and 1 with a compound
mutation. Three subjects were not tested and 3 subjects had uncharacterized mutations. Among those who had genetic testing, there were differences in genetic results in Group 1 vs. Group 2 subjects (Table 1). For example, 83% of Group 1 subjects had a \(KCNQ1\) or \(KCNE1\) mutation, 4% had an \(SCN5A\) mutation and no subject had an uncharacterized mutation. In contrast, 33% of Group 2 subjects had a \(KCNQ1\) mutation, 33% had an \(SCN5A\) mutation and nearly 20% had uncharacterized mutations (n = 3).

**Fetal heart rates**

Normal FHR data from 7-40 weeks was obtained from 3,264 FHR measurements in 547 normal subjects. The 3\(^{rd}\), 50\(^{th}\), and 97\(^{th}\) percentiles for GA are shown in **Figure 1**. We obtained 318 FHR measures from 42 LQTS fetuses; the mean of FHR measures was ~8/fetus and the range was 01 to 12/fetus. The mean FHR at each of the 3 GA groups (< 21 weeks, 21-30 weeks, and 31-40 weeks) was significantly different between normal and LQTS subjects \((P < 0.001)\) (**Table 2**). The FHR at the 3\(^{rd}\) percentile of normal from the three GA groups were all greater than the standard obstetrical definition of fetal bradycardia, i.e. FHR ≤ 110 bpm.

We evaluated the individual FHR measures across GA of fetuses based on indication for referral. **Figure 2A** shows the FHR/GA profile of subjects referred for a family history; **Figure 2B** shows the FHR/GA profile of subjects referred for evaluation of fetal rhythm. The FHR of the LQTS fetuses decreased with GA as seen in the normal fetal cohort, but GA dependent changes in mean FHR differed between Groups 1 and 2. Early, at < 21 weeks of gestation, mean FHRs were not different \((141.54 \pm 2.02 \text{ vs. } 136.95 \pm 2.51; \ P = 0.15)\). However, FHR in Group 2 was lower at both 21-30 weeks \((130.28 \pm 2.66 \text{ vs. } 124.54 \pm 2.65; \ P = 0.04)\) and at 31-40 weeks \((127.72 \pm 2.81 \text{ vs. } 117.94 \pm 2.83; \ P < 0.01)\). Only subjects in Group 2 had FHR ≤ 110 bpm.

**The bradycardia index**
Among LQTS subjects, only 15% of FHR readings were \( \leq 110 \text{ bpm} \) while 66% of the FHR readings were \( \leq 3^{\text{rd}} \) percentile for GA. Thus, 85% of the total FHR readings were higher than the standard obstetrical definition of bradycardia (FHR \( \leq 110 \text{ bpm} \)), and only 33% of the LQTS FHR readings were \( > 3^{\text{rd}} \) percentile for GA. Using FHR \( \leq 3^{\text{rd}} \) percentile for GA, 38% (16/42) of LQTS fetuses had a bradycardia index of 100% and 67% (28/42) had a bradycardia index between 75-100%. Table 3 shows there were significant differences between Groups 1 and 2 in the bradycardia indices for FHR \( \leq 110 \text{ bpm} \) and \( \leq 3^{\text{rd}} \) percentile for GA. Within Group 2, a bradycardia index of 100% was seen in 2/3 subjects with complex rhythms (Table 4). The findings of more pronounced bradycardia in Group 2 subjects with complex rhythms may be another manifestation of a more severe phenotype in such fetal LQTS subjects.

Although the sample sizes of certain LQTS mutations were small, among genotypes, the bradycardia index for FHR \( \leq 3^{\text{rd}} \) percentile for GA was highest (100%) for uncharacterized mutations and lowest (0%) for LQT5 mutations. Overall, the severity of the bradycardia index was not predicted by the presence of mutations in known LQTS genes.

Fetal Heart Rhythms and FHR

In most of the 42 fetuses, sinus rhythm was observed throughout pregnancy, but in 10 fetuses, 8 of whom were in Group 2, complex arrhythmias characterized by 2\(^{\circ}\) AV block and/or TdP, were observed (Table 4). The mean FHRs of these 10 subjects were lower across GA than those subjects who manifested only fetal bradycardia (120.74 ± 3.56 vs. 130.79 ±2.37; \( P < 0.01 \)) and the bradycardia indices for FHR \( \leq 3^{\text{rd}} \) percentile were higher (80% vs. 60%).

Discussion

There are several novel and clinically relevant findings in this study of fetal LQTS. First, as in
normal fetuses, the FHR of LQTS subjects trend downward but are generally lower than FHR of normal fetuses as gestation progresses. Second, there are GA dependent FHR predictors of LQTS; for example, when compared to a GA independent FHR predictor (FHR ≤ 110 bpm), a FHR ≤ 3rd percentile for GA improves ascertainment of LQTS subjects from 15 to 85%. Third, there are “shades of bradycardia” within the LQTS population: compared to subjects who remained in sinus rhythm during pregnancy, subjects with the lowest FHRs were more likely to have had a complex arrhythmia including TdP and/or 2° AV block, and were more likely to have de novo or uncharacterized mutations. Together, findings from this study should improve ascertainment of fetal LQTS at all GA.

From the first ultrasound visualization of the fetal heart signifying a viable pregnancy to the reactive accelerations signifying fetal well-being during labor and delivery, FHR is the most frequently and thoroughly evaluated parameter from the beginning to the end of pregnancy. Yet, neither the range of normal nor the definitions of abnormal FHR are GA specific, emphasizing the shortcomings of a single definition of fetal bradycardia, e.g. FHR ≤ 110 bpm. Sinus bradycardia occurs in LQTS, but the molecular basis for this common occurrence is incompletely understood. Furthermore, the GA at which the sinus beat becomes bradycardic, and indeed the sensitivity and specificity of a GA independent definition of bradycardia, are poorly understood.

Previous publications have described a range of sinus FHRs ranging from < 100 to 130 bpm in LQTS fetuses. As in our series, many of these LQTS fetuses with FHRs in the “normal range” (> 110 bpm) had a family history of LQTS. The higher FHR in those with a family history may be ascertainment bias as these subjects, screened preemptively may be less severely affected. After birth, the majority of subjects, even those with FHR ≤ 110 bpm, had heart rates in the “normal range” 7. Similarly, in a large study evaluating LQTS and SIDS,
“bradycardia” in the neonate was not considered a risk factor for LQTS. Thus, use of a stringent fetal bradycardia definition, *i.e.* FHR ≤ 110 bpm, may result in failure to recognize fetal LQTS, and continuation of “mild” bradycardia, *i.e.* heart rate > 110 bpm, after birth may fail to raise the suspicion of LQTS in the neonate. This may explain why the older siblings of some fetal probands in this study with mild bradycardia, were not suspected as fetuses or neonates to have LQTS.

In the absence of a known family history, the ascertainment of fetal LQTS is based on the correct and timely diagnosis of the signature LQTS rhythms. While TdP and 2° AV block are usually easily recognized and have high specificity, they occur infrequently in the fetus with LQTS. For example, only 24% of our study cohort had these complex arrhythmias, and none of the 25 subjects in this report with LQT1 mutations had TdP or 2° AV block. Thus, it is important to identify other markers of LQTS; findings in our study suggest that FHR may be useful for this purpose. Our results show that a “one size fits all” FHR indicator of bradycardia will not be adequate. For example, the bradycardia index of LQT1 subjects for FHR ≤ 110 bpm was only 2% but definition of bradycardia as FHR ≤ 3rd percentile yielded a bradycardia index of 68%. Using a GA independent definition of bradycardia would not have led to suspicion of LQTS in many such subjects.

Although our study group is relatively small, we found associations between FHR, rhythm phenotype, and genotype which could be helpful in the diagnosis of LQTS. For example, individuals with *KCNQ1* mutations tended to have a mild phenotype *in utero* with sinus rhythm and mild bradycardia. On the other hand, genetically elusive subjects, with no known mutations and a negative family history of LQTS, had profound fetal bradycardia and complex rhythms.

Based on the results of this study, we believe that FHR ≤ 3rd percentile for GA is a
superior definition of fetal bradycardia compared to the widely used obstetrical definition. Our study suggests that the fetus with repeated FHR measurements ≤ 3rd percentile for GA without any other rhythm abnormality should be suspected of having LQTS. This suspicion should lead to detailed family history for LQTS. Regardless of family history, a postnatal 12-lead ECG should be examined for findings of LQTS. If the family history is positive, or the fetal proband manifests complex LQTS rhythms, ECG screening of first-degree relatives is recommended. Even if family members are asymptomatic, ECG evidence of LQTS warrants genetic testing. Finally, if postnatal genetic testing of the fetus with suspected LQTS is positive, but clinical and/or genetic manifestations of LQTS are negative in first-degree relatives, the possibility of parental mosaicism should be considered, especially if future pregnancies are contemplated. 

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Conflict of Interest Disclosures: None.

References:


34. Murphy LL, Moon-Grady AJ, Cuneo BF, Wakai RT, Yu S, Kunic JD, Benson DW, George...


Table 1. Study Cohort.

<table>
<thead>
<tr>
<th>Group 1: Referral for Family History of LQTS</th>
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<tr>
<th>Group 2: Referral for Fetal Arrhythmia</th>
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<td>42</td>
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Key: Numbers in parenthesis following subject number are references in which subject was previously described; numbers in parenthesis following mutations are references for first description of mutation. No. = number; GA = gestational age; FHR = fetal heart rate; wks = weeks; * indicates mother with LQTS was on beta blocker treatment during entire pregnancy; † mother on beta blocker therapy only during 3rd trimester; ‡ novel mutation; F/N = fetus/neonate; IUFD = intrauterine fetal demise.
Table 2. Mean FHR of Normal and LQTS Subjects by GA Group.

<table>
<thead>
<tr>
<th>GA Group</th>
<th>Normal Subjects FHR mean ± SE (bpm)</th>
<th>LQTS Subjects FHR mean ± SE (bpm)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 21 weeks</td>
<td>152.0 ± 0.4</td>
<td>139.9 ± 1.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>21-30 weeks</td>
<td>146.7 ± 0.2</td>
<td>127.4 ± 1.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>31-40 weeks</td>
<td>143.3 ± 0.4</td>
<td>123.0 ± 1.4</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Key: GA = gestational age; FHR = fetal heart rate; SE = standard deviation; bpm = beats per minute.

Table 3. Bradycardia Index of Group 1 and Group 2 Based on GA Group.

<table>
<thead>
<tr>
<th>GA (weeks)</th>
<th>FHR ≤ 110 bpm</th>
<th>FHR &lt; 3rd percentile for GA</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Group 1</td>
<td>Group 2</td>
</tr>
<tr>
<td>overall</td>
<td>1%</td>
<td>31%</td>
</tr>
<tr>
<td>&lt; 21</td>
<td>0%</td>
<td>6%</td>
</tr>
<tr>
<td>21-30</td>
<td>2%</td>
<td>26%</td>
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<tr>
<td>31-40</td>
<td>2%</td>
<td>49%</td>
</tr>
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Key: GA = gestational age; FHR = fetal heart rate; bpm = beats per minute.

Table 4. Complex Fetal Rhythms in Relation to Bradycardia Index in LQTS Cohort.

<table>
<thead>
<tr>
<th>ID</th>
<th>LQTS Mutation</th>
<th>Fetal Rhythm</th>
<th>% FHR readings ≤ 3rd percentile GA</th>
<th>% FHR ≤ 110 bpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Untested</td>
<td>2° AVB</td>
<td>100</td>
<td>100%</td>
</tr>
<tr>
<td>17</td>
<td>KCNQ1-G168R</td>
<td>2° AVB</td>
<td>100</td>
<td>0%</td>
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Group 2: Referral for Fetal Arrhythmia

<table>
<thead>
<tr>
<th>ID</th>
<th>LQTS Mutation</th>
<th>Fetal Rhythm</th>
<th>% FHR readings ≤ 3rd percentile GA</th>
<th>% FHR ≤ 110 bpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>27</td>
<td>KCNH2-G628S</td>
<td>TdP and 2° AVB</td>
<td>100</td>
<td>84%</td>
</tr>
<tr>
<td>28</td>
<td>SCN5A-R1623Q</td>
<td>TdP and 2° AVB</td>
<td>100</td>
<td>75%</td>
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<tr>
<td>30</td>
<td>Uncharacterized</td>
<td>2° AVB</td>
<td>100</td>
<td>92%</td>
</tr>
<tr>
<td>33</td>
<td>KCNQ1-G314D</td>
<td>2° AVB</td>
<td>86</td>
<td>0%</td>
</tr>
<tr>
<td>36</td>
<td>SCN5A-R1623Q</td>
<td>TdP and 2° AVB</td>
<td>71</td>
<td>14%</td>
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Key: TdP = torsade de pointes; AVB = atrioventricular block; GA = gestational age; FHR = fetal heart rate.
Figure Legends:

**Figure 1.** Individual fetal heart rate (FHR) measurements (n = 3264 data points) by gestational age of 547 normal fetuses. Curves representing the 3rd, 50th, and 97th percentiles of FHR are shown, as is a horizontal line at 110 bpm, which is the standard obstetrical definition of bradycardia. FHR decreases with advancing gestational age. Some normal FHR measurements are < 3rd percentile but none are < 110 bpm.

**Figure 2.** Individual FHR measurements throughout gestation of LQTS fetuses, based on indication for referral. Panel A shows the FHR of individuals referred because of a family history of LQTS (Group 1). Panel B shows the FHR of individuals referred for evaluation of fetal rhythm (Group 2). For reference, the line marking a FHR of 110 bpm across gestation is shown in both panels.
Fetal Heart Rate Predictors of Long QT Syndrome
Jason L. Mitchell, Bettina F. Cuneo, Susan P. Etheridge, Hitoshi Horigome, Hsin-Yi Weng and D. Woodrow Benson

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SUPPLEMENTAL MATERIAL

Expanded Methods

Animal model and hypoxic exposure

Eight to 10-week old mice (FVB strain) were housed in large plexiglass chambers and the FiO₂ regulated by an OxyCycler controller (BioSpherix, Redfield, NY). Hypoxic exposures were performed at 8.5±0.5 % O₂ and ventilation was adjusted to insure that CO₂ levels did not exceed 5,000 ppm (average range 1,000-3,000 ppm). Ammonia was removed by ventilation and activated charcoal filtration through an air purifier. Under these conditions, the ammonia concentration is less than 2.5 ppm, the limit of detection of Gastec passive dosimetry tubes (Sigma). Animals were anesthetized with pentobarbital (50 mg/kg, I.P.) and injected through the left jugular vein with concentrated conditioned media (5 μg protein in 50 μl) or exosome preparations (0.1 μg protein in 50 μl PBS). A higher dose of exosomes (10 μg protein in 50 μl PBS) was delivered through the tail vein. Table 1 lists the number of cells used and the exosomal protein recovery from each cell type. Approximately 2% of secreted proteins in the conditioned media of both mMSCs and MLFs are associated with the exosomal fraction and roughly 3-fold more MLFs were required to extract equal amounts of exosomes for the injections. An equal volume of PBS or serum-free α-MEM media was injected in control experimental groups. Mice were allowed to recover for 3 hours before placement in hypoxic chambers. In certain time-course and dose-dependent studies, a second injection of MEX (0.1 μg protein in 50 μl PBS) was performed on the contralateral jugular vein after 4 days of hypoxic exposure.
Isolation of human MSCs from umbilical cord Wharton’s Jelly

Human umbilical cord Wharton’s jelly derived MSCs (hUC-MSCs) were isolated according to published methods\textsuperscript{1,2} with minor modifications. Cord was rinsed twice with cold sterile PBS, cut longitudinally, and arteries and vein were removed. The soft gel tissues were scraped out, finely chopped (2-3 mm\textsuperscript{2}) and directly placed on 100 mm dishes (15 pieces per dish) with DMEM/F12 (1:1) (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone), 2 mM L-glutamine, and penicillin/streptomycin, and incubated for 5 days at 37°C in a humidified atmosphere of 5% CO\textsubscript{2}. After removal of tissue and medium, the plates were washed 3 times with PBS, the attached cells were cultured and fresh media replaced 3 times per week. At 70-80% confluence, cells were collected and stained with PE conjugated antibodies for CD34 (Miltenybiotec, Auburn, CA) and CD45 (Miltenybiotec, Auburn, CA). Immunodepletion was performed using the anti-PE-microbeads (Miltenybiotec, Auburn, CA) and MSCS column (Miltenybiotec, Auburn, CA) according to manufacturer’s instructions. The CD34 and CD45 negative populations were further propagated and selected for the expression of MSC markers (CD105, CD90, CD44, and CD73) and the absence of CD11b, CD19, and HLA-DR by using a set of fluorescently-labeled antibodies designed for the characterization of human MSCs (BD Biosciences, San Diego, CA) and a MoFlo flow cytometry (Beckman Coulter).

Cell culture and collection of conditioned media

Bone marrow-derived MSCs, isolated from the femurs and tibiae of 5-7 week old male FVB mice, were selected and their differentiation potential assessed as previously described\textsuperscript{3}. Briefly, after 3-4 passages, plastic adherent cells were immunoselected using mouse specific antibodies (BD Biosciences Pharmingen, San Diego, CA) and a MoFlo fluorescence-activated cell sorter (FACS) (Dakocytomation, Fort Collins, CO), as
we reported previously\(^3,4\) in compliance with published MSC criteria\(^5\). Cells were negatively selected for CD11b, CD14, CD19, CD31, CD34, CD45, and CD79\(\alpha\) antigens, and positively selected for CD73, CD90, CD105, c-kit and Sca-1 antigens. Primary MLF cultures were derived according to standard methods\(^6,7\).

To exclude contamination from serum-derived microvesicles, serum used for propagation of cell cultures and the collection of CM was clarified by ultracentrifugation at 100,000 \(x\) \(g\) for 18 hours. MSCs were cultured in \(\alpha\)-MEM media supplemented with 10% (v/v) FBS (Hyclone), 10% (v/v) Horse Serum (Hyclone), 2 mM L-glutamine (GIBCO), and antibiotics. MLFs were cultured in DMEM (Invitrogen) supplemented with 10% (v/v) FBS and 2 mM L-glutamine. Cultures at 70% confluence were washed twice with PBS and incubated with serum-free media supplemented with 2 mM L-glutamine for 24 hours under standard culture conditions. Conditioned media were collected and cells and debris were removed by differential centrifugations at 400 \(x\) \(g\) for 5 mins, at 2,000 \(x\) \(g\) for 10 mins, and at 13,000 \(x\) \(g\) for 30 mins. The clarified CM were subsequently filtered through a 0.2 \(\mu\)m filter unit and concentrated using an Ultracel-10K (Millipore) centrifugal filter device, to a protein concentration range of 0.1-0.5 mg/ml. Protein levels in the CM were determined by Bradford assay (Bio-Rad, Hercules, CA).

**Bronchoalveolar lavage**

Animals were anesthetized with Avertin (250 mg/Kg \(i.p.\)) and their trachea cannulated with a blunt-ended 20 gauge Luer Stub Adapter (Becton Dickinson). BALF was collected via sequential administration of PBS supplemented with 5 mM EDTA (0.8 ml, 0.8 ml, 0.8 ml, and 0.9 ml) and approximately 3.0 ml (+/- 0.1 ml) of BALF was recovered.
per animal. Cells in BALF were collected by centrifugation at 400 xg for 10 min and leukocytes stained with Kimura solution for counting.

**Right ventricular systolic pressure measurements**

Mice were anesthetized with 60 mg/kg of pentobarbital and remained spontaneously breathing. A small incision was made in the abdominal wall, and the translucent diaphragm exposed. A 23-gauge butterfly needle with tubing attached to a pressure transducer was inserted through the diaphragm into the right ventricle and pressure measurements were recorded with PowerLab (ADInstruments, Colorado Springs, CO) monitoring hardware and software. Animals with heart rates less than 300 beats per minute were considered over-anesthetized and their RVSP measurements were excluded. Mean RVSP over the first ten stable heartbeats was recorded.

**Right ventricular weight measurements**

Hearts and pulmonary vasculature were perfused *in situ* with cold 1X PBS injection into the right ventricle; hearts were excised and used for Fulton’s Index measurements (ratio of RV weight over left ventricle plus septal weight, RV/[LV+S]). Both ventricles were weighed first, then the right ventricular free wall was dissected and the remaining LV and ventricular septum was weighed.

**Pulmonary histology**

Lungs were inflated by tracheotomy and perfused with 4 % paraformaldehyde, excised, and fixed in 4 % PFA overnight at 4°C followed by paraffin embedding. Sections (two per animal) from 4 individuals in each group (group n > 7) were analyzed for pulmonary histology. For pulmonary vascular morphometry, paraffin-embedded lung sections were stained with hematoxylin and eosin. For immunohistochemical analysis, 5 µm lung
tissue sections were deparaffinized in xylene and rehydrated. Tissue slides were treated with 0.3% \( \text{H}_2\text{O}_2 \) in methanol to inactivate endogenous peroxidases and blocked with horse serum for 1 hour. After incubating with monoclonal anti-mouse \( \alpha \)-SMA antibody (Sigma) at a dilution of 1:125 overnight at 4\(^\circ\)C, secondary antibodies and peroxidase staining was applied according to manufacturer’s instructions (Vector Laboratories, Burlingame, CA). Vessel wall thickness was assessed by measuring \( \alpha \)-SMA staining in vessels (20-40 \( \mu \text{m} \) in diameter) within each field (40-50 fields per section) captured at 400X magnification with a microscope digital camera system (Nikon, Tokyo, Japan), and using Metamorph image analysis program (Molecular Devices, Sunnyvale, CA). The medial wall thickness index was calculated by the following formula: Wall thickness (%) = 100 x (area[ext] – area[int]) / area[ext] where area[ext] and area[int] denote the areas bounded by the \( \alpha \)-SMA layer.

**In vitro hypoxia**

Human PAECs were purchased from GIBCO and cultured in M200 medium (Invitrogen) supplemented with LSGS (Invitrogen). At 80% confluence, cells were exposed to 1% \( \text{O}_2 \) for 6 hours in an inVivo\( _2 \) workstation (Ruskin Technology, Bridgend, UK) in the presence or absence of exosomal fraction (1 \( \mu \text{g/ml} \)), or the exosome-depleted fraction of hUC-MSC conditioned media (1 \( \mu \text{g/ml} \)). Cells were lysed and proteins in whole cell lysates were separated on 8% SDS-polyacrylamide gel electrophoresis followed by western blot analysis using rabbit monoclonal antibody for phospho-STAT3 (Y705) and mouse monoclonal STAT3 antibody (Cell Signaling).
Electron microscopic analysis

EM analysis was performed at the Harvard Medical School electron microscope facility. Exosome preparations were adsorbed to a carbon coated grid that had been made hydrophilic by a 30 second exposure to a glow discharge. Excess liquid was removed and the samples were stained with 0.75% uranyl formate for 30 seconds. After removing the excess uranyl formate, the grids were examined in a JEOL 1200EX Transmission electron microscope and images were recorded with an AMT 2k CCD camera.

Protein extraction and immunoblotting

BALF (3 ml) was centrifuged at 420 x g for 10 min and cell-free BALF supernatants were used for protein analysis. Equal volumes of BALF specimens from individual animals in the same group were pooled (1 ml) and proteins precipitated overnight by 20% trichloroacetic acid (Sigma). A fraction equivalent to 30% of each protein pellet was dissolved in 1x sodium lauryl sulfate (SDS)-loading buffer was separated on a denaturing 15% polyacrylamide gel. After transfer to 0.2 µm PVDF membranes (Millipore), blots were blocked with 5% skim milk and incubated with 1:1,000 diluted rabbit polyclonal MCP-1, galectin-3, or HIMF/FIZZ1 antibody (Abcam) for overnight at 4°C. To detect mouse Immunoglobulin A, 1:5,000 diluted goat anti-mouse IgA antibody (Abcam) was used. Peroxidase-conjugated anti-rabbit secondary antibody (Santa Cruz Biotech) was used in 1:20,000 dilution to visualize immunoreactive bands either by the enhanced chemiluminescence reagent (Pierce) or Lumi-LightPLUS (Roche).

For analysis of proteins from whole lung, frozen lung tissues were homogenized for 5 seconds with Polytron in cold PBS containing 2 mM phenylmethanesulfonyl fluoride
(Sigma) and centrifuged at 3,000 xg for 3 mins. Tissue pellets were washed twice with cold PBS containing 2 mM PMSF followed by centrifugation at 3,000 xg for 3 mins and lysed in RIPA buffer containing protease inhibitor (Roche) and phosphatase inhibitor cocktails (Thermo). Forty μg of lung tissue extracts were separated on 10-20% gradient gel (Invitrogen). Antibodies for MCP-1, HIMF, IL-6, STAT3, and phospho-STAT3 (Y705) were used for immunoblotting. For loading control, mouse monoclonal β-actin antibody (Sigma) was used.

Proteins in exosome preparations were separated on 12% polyacrylamide gel and then transferred onto 0.45 μm PVDF membrane (Millipore). Goat polyclonal anti-CD63 antibody (Santa Cruz Biotech), mouse monoclonal Alix and TSG101 antibodies (Santa Cruz Biotech), rabbit polyclonal CD81, CD9, hsp90, and flotillin-1 antibodies (Santa Cruz Biotech), and rabbit polyclonal Dicer (Abcam) antibody were used for immunoblotting.

**Isolation and Quantification of microRNAs**

Total lung RNA was extracted by the method of Chomczynski & Sacchi and 750 ng was used as a template for reverse transcriptase with specific primers for each target microRNA (TaqMan Reverse Transcription Kit, Applied Biosystems, Foster City, CA). Each reverse transcription reaction included also the primer for the small nuclear RNA sno202, which was used as an internal control. 37.5 ng cDNA was used for each 20 μl qPCR reaction with TaqMan universal master mix II with no UNG (Applied Biosystems) in the presence of probes specific for the indicated microRNAs and the internal control (TaqMan microRNA assay, Applied Biosystems). Amplification was performed at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 1 min, on a StepOne Plus platform (Applied Biosystems).
RNAs from isolated exosomes were extracted by Trizol reagent (Invitrogen). Briefly, 30 µg exosomal protein was mixed with 0.5 ml Trizol reagent per manufacturer’s recommendation. 20 µg RNase-free glycogen (Ambion) was applied as a carrier prior to RNA precipitation with isopropyl alcohol and samples were placed at -80 °C for overnight. 150 ng exosomal RNAs were used as a template in reverse transcription reactions with specific primers for target microRNAs (TaqMan Reverse Transcription Kit, Applied Biosystems). To quantify pre-let7b, 300 ng of exosomal RNAs were reverse transcribed by High Capacity RNA-to-cDNA kit (Applied Biosystems) per manufacturer’s recommendations. 7.5 ng of cDNA for each microRNA assay and 11.5 ng of cDNA for pre-let7b (TaqMan gene expression assay, Applied Biosystems) were used for qPCR reaction in the presence of specific probes. Amplification was performed as described above. Let7a was used as an internal control.

**Smooth Muscle Cell Proliferation Assay**

Primary rat PASMCs were inoculated at a concentration of 2 x 10³/well on a 96 well plate in DMEM containing 5% FBS and incubated for 24 hours under standard culture conditions. After serum starvation for 2 days in 0.1% FBS/DMEM, cells were pretreated either with vehicle or varying doses of mMEX (16, 31.5, 62.5, 125 ng/ml) for 30 min then FBS was added at 5% (v/v) to each well. After incubation for 48 hours, cell proliferation reagent WST-1 (Roche), which is cleaved by mitochondrial dehydrogenases in metabolically active cells to form formazan dye, was directly applied to the cells followed by further incubation for 3 hours. Intensity of solubilized dark red formazan was determined at 440 nm using a microplate reader.
Supplemental References


## Quantities of exosomes from cultures of mMSCs and MLFs

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**Supplemental Table 1**
**Supplemental Figure Legends**

**Supplemental Figure 1**
Flow cytometric analysis of surface-markers for human MSCs. Human MSCs from umbilical stroma (hUC-MSCs) were cultured in DMEM/F-12(1:1) supplemented with 10% FBS. Human UC-MSCs at passage 5 express CD90, CD105, CD73, CD44, but lack expression of CD19, CD34, CD45, CD11b, and HLA-DR in flow cytometric analysis. Isotype-matched IgG controls are shown with non-shaded dotted curves, and hUC-MSCs curves are shown in red shaded area.

**Supplemental Figure 2**
Comparison of exosomes from two methods of isolation. Exosomes in the medium conditioned by mMSCs were isolated by ultracentrifugation (UCF) or S200 size-exclusion chromatography (SEC). Two μg of proteins from each preparation were loaded onto 12% SDS-PAGE and total proteins stained by SimplyBlue (Invitrogen) (left panel). Exosomal markers, HSP90, flotillin-1, and CD63 were detected by immunoblotting and are comparable in both preparations (right panel).

**Supplemental Figure 3**
Dose-dependent inhibition of SMC proliferation by MEX. Cultured rat PASMCs were serum-deprived for 48 hours followed by treatment with mMEX (16 to 125 ng/ml) in the presence of 5% FBS and their proliferation rate was quantified relative to the treatment with FBS alone. Data are expressed as mean values ± SD. *, p < 0.001 vs. FBS-alone (One-way ANOVA with Tukey-Kramer post-test).
Supplemental Figure 4

MicroRNA content in mouse MEX compared with FEX. RNAs extracted from equivalent amount of MEX and FEX were subjected to RT-qPCR analysis. Levels of the indicated microRNAs relative to let7a in MEX and FEX are presented on the left panel and comparisons between the level of pre-let7b and let7b relative to let7a in MEX and FEX are shown on the right. Data are presented as mean values ±SD. *, p < 0.001 MEX vs. FEX (Student's t-test).

Supplemental Figure 5

Schema of a hypothesis synthesizing the results of this study with our previous work and published literature. Hypoxia shifts the Th1/Th2 balance of immunomodulators in the lung, resulting in alternative activated alveolar macrophages (AA-AMΦ) and, in the early phase, induces the expression of IL-6, MCP-1, and HIMF in the lung epithelium. HIMF mitogenic action on the vasculature requires Th2 cytokines, such as IL-4. Consequences of the shift towards proliferation include the hypoxic activation of STAT3 signaling and the upregulation of the miR-17 family of microRNAs. Treatment with MEX interferes with an early hypoxic signal in the lung, suppressing both inflammation and HIMF transcriptional upregulation. It addition, MEX treatment may directly upregulate miR-204 levels, thus breaking the STAT3-miR-204-STAT3 feed-forward loop, and shifting the balance to an anti-proliferative state.
Supplemental Figure 1
Supplemental Figure 2
Supplemental Figure 3
Supplemental Figure 5