Hyaluronidase 2 Deficiency Causes Increased Mesenchymal Cells, Congenital Heart Defects, and Heart Failure

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Background—Hyaluronan (HA) is required for endothelial-to-mesenchymal transition and normal heart development in the mouse. Heart abnormalities in hyaluronidase 2 (HYAL2)–deficient (Hyal2−/−) mice and humans suggested removal of HA is also important for normal heart development. We have performed longitudinal studies of heart structure and function in Hyal2−/− mice to determine when, and how, HYAL2 deficiency leads to these abnormalities.

Methods and Results—Echocardiography revealed atrial enlargement, atrial tissue masses, and valvular thickening at 4 weeks of age, as well as diastolic dysfunction that progressed with age, in Hyal2−/− mice. These abnormalities were associated with increased HA, vimentin-positive cells, and fibrosis in Hyal2−/− compared with control mice. Based on the severity of heart dysfunction, acute and chronic groups of Hyal2−/− mice that died at an average of 12 and 25 weeks respectively, were defined. Increased HA levels and mesenchymal cells, but not vascular endothelial growth factor in Hyal2−/− embryonic hearts, suggest that HYAL2 is important to inhibit endothelial-to-mesenchymal transition. Consistent with this, in wild-type embryos, HYAL2 and HA were readily detected, and HA levels decreased with age.

Conclusions—These data demonstrate that disruption of normal HA catabolism in Hyal2−/− mice causes increased HA, which may promote endothelial-to-mesenchymal transition and proliferation of mesenchymal cells. Excess endothelial-to-mesenchymal transition, resulting in increased mesenchymal cells, is the likely cause of morphological heart abnormalities in both humans and mice. In mice, these abnormalities result in progressive and severe diastolic dysfunction, culminating in heart failure. (Circ Cardiovasc Genet. 2017;10:e001598. DOI: 10.1161/CIRCGENETICS.116.001598.)

Key Words: cor triatrium • developmental biology • endocardium • extracellular matrix • live birth
Dеградация HA предполагается быть завершенной в соматических клетках HYAL1 и HYAL2. HYAL2 является гликолипидно-связанным протеином гликолипидной мембраны (GPI). HYAL2 имеет слабую активность по отношению к HA, а его цистерны, содержащие фрагменты массы HA, приводят к образованию соматических клеток. HYAL2 участвует в антифөнуксисе HA, вызывая интенсивное образование HA в соматических клетках. HYAL2 является ключевым фактором для уменьшения EMT и мезенхимальной клеточной пролиферации.

Структура и функция сердца в Hya2-/- и контрольных мышах были изучены с помощью эхокардиографии. Сильное расширение предсердий, сопровождающееся диастолической дисфункцией, было обнаружено в Hya2-/- мышах. Гистологические исследования показали, что расширение предсердий в Hya2-/- мышах происходит из-за избытка клеток мезенхимы и не коррелирует с присутствием кора триатриума. Hya2-/- мыши имели более высокое количество мезенхимальных клеток во время развития, указывая на увеличение EMT или мезенхимальной клеточной пролиферации. Эти наблюдения говорят о том, что HYAL2 необходим для подавления EMT и мезенхимальной клеточной пролиферации при присутствии HA. Эти данные подчеркивают важность HYAL2 в управлении гомеостазом HA в организме.

Материалы и методы

Мышь. Мышь, у которой нет HYAL2, Hya2-/- мыши, были выведены на свет в течение 4 недель, и с возрастом произошло ухудшение, включая развитие структурных мутаций сердца, таких как атриовентрикулярная недостаточность, при которой 100% мышей Hya2-/- имели толстые стернальные и межстенальные стенки, а также предсердные расширения (50%), атриовентрикулярные недостаточности (50%) и нарушения клапанов. Дополнительно, были выявлены другие мутации, такие как нерождённые (только 33% выживали на росте), прекрашённые (только 9% выживали на росте) и мутации, при которых 100% мышей Hya2-/- умирали до рождения (5% мышей Hya2-/- умирали до рождения).

Микро-Компьютерная томография

Харты были исследованы с помощью микротомографа Skyscan 1176 с использованием раствора фосфотунгстата в концентрации 1% для удаления растворимых HA. Сканы были выполнены с шагом 0.5 μm, и полученные данные анализировались с помощью программного обеспечения Skyscan Micro-CT CT-Analyser Version 1.13. Реконструкция 3D-изображений была выполнена с использованием программного обеспечения Bruker Micro-CT CT-Analyser Version 1.13. Статистический анализ

Данные представлены в виде M±SEM. Статистический анализ проводился с помощью теста t и критерия Пирсона. Для каждого сердечного фенотипа с повторными измерениями, были проведены сравнения между группами, и значения p<0.05 считались значимыми. Для каждого сердечного фенотипа с повторными измерениями, были проведены сравнения между группами, и значения p<0.05 считаются значимыми.
Results

Atrial Enlargement in Hyal2−/− Mice

To understand the developmental origins of the cardiac abnormalities in Hyal2−/− mice, we conducted a longitudinal analysis of cardiac structure and function at 4-week intervals beginning at 4 weeks of age using high-frequency ultrasound. At 4 weeks of age, all Hyal2−/− mice exhibited significant atrial enlargement (Figure 1A through 1C, dashed lines). The most severely affected mice reached a humane end point at an average of 9 weeks, 15 weeks earlier than their less severely affected Hyal2−/− littermates. The severe atrial enlargement in 50% of mice was consistent with our previous study in which 54% of mice were found to have severe atrial enlargement at death.5 On the basis of these findings, severely affected Hyal2−/− mice were deemed acute, and less severely affected Hyal2−/− mice were deemed chronic; these 2 groups were analyzed independently throughout the study. Quantification of the atrial enlargement by measuring the diameter of the atrium from 2-dimensional ultrasound images revealed a 1.8-fold increase in the acute Hyal2−/− mice at 4 weeks of age compared with controls and a 1.3-fold increase in acute compared to chronic Hyal2−/− mice (Figure 1G).

Ultrasound imaging revealed increased tissue density in the atria of Hyal2−/− mice (Figure 1D through 1F), which in the acute group blocked the view of the atrium (Figure 1D) that was normally clearly visible (Figure 1E and 1F). No progressive change in the size of the atrium was detected in the Hyal2−/− mice (Figure 1G). We were unable to measure the ventricular diameter because the apex of the heart could not be reproducibly visualized in the ultrasound images. However, there were no instances where the ventricles were grossly distended like the atria of Hyal2−/− mice.

Valve Thickening in Hyal2−/− Mice

We previously showed that all heart valves were thickened in adult Hyal2−/− mice.5 However, whether this thickening occurred before or after birth was unknown. Brightness-mode images revealed significantly thickened valves were already present at 4 weeks of age in Hyal2−/− mice and did not change significantly with age (Figure 1H and 1I). No significant difference was found in the valve thickness of acute and chronic Hyal2−/− mice. Brightness-mode imaging allowed the measurement of only the aortic and mitral valves, which we used as a proxy for valve thickness in general (Figure 1H and 1I).

Figure 1. Structural abnormalities in hyaluronidase 2 (HYAL2)–deficient (Hyal2−/−) mouse hearts. A–C, High-resolution micro-computed tomographic images of Hyal2−/− and control hearts. Micro-computed tomographic images were reconstructed in 3-dimensional and colorized to enhance structural visualization of the atria (dashed lines). Hyal2−/− mice with a grossly enlarged atrium (A) were deemed acute, whereas those with a mildly enlarged atrium (B) were deemed as chronic. A heart from a control mouse is shown in (C). D–F, Ultrasound images of Hyal2−/− and control hearts. Brightness-mode images of the heart showed an enlarged and dense left atrium (dashed lines) in the acute Hyal2−/− mice compared with chronic Hyal2−/− and control mice. The increased density is indicated in the image by the stronger white signal. G, Atrium diameter in Hyal2−/− and control mice. The diameter of the atrium was significantly larger in both acute and chronic Hyal2−/− mice compared with controls. *, †, ‡ P<0.05; **, ††, ‡‡ P<0.01; §§, †††, ‡‡‡, §§§ P<0.001. The number of animals used were for atrial enlargement control and chronic (n=6); acute (at 1 mo n=7; 2 mo n=4; and 3 mo n=3), for AV thickness control and chronic (n=6), acute (at 1 mo n=7; 2 mo n=4, and 3 mo n=3), for MV thickness control and chronic (n=6), acute (at 1 mo n=6; 2 mo n=4, and 3 mo n=3).
in the Data Supplement). Interestingly, histological studies of the heart valves at postnatal day 1 (P1) revealed only minimal valve thickening in *Hyal2*−/− mice, suggesting that thickening occurred during early postnatal valve remodeling.

**Heart Function in *Hyal2*−/− Mice**

To determine how the structural abnormalities affected cardiac function in *Hyal2*−/− mice, we analyzed several parameters using echocardiography. The peak velocities of early to late atrial filling of the left ventricle were inverted in *Hyal2*−/− mice (Figure II in the Data Supplement) and resulted in a significantly reduced early to late atrial filling ratio in all *Hyal2*−/− mice compared with controls (Figure 2A). In the acute *Hyal2*−/− mice, the early to late atrial filling ratio was significantly lower at 3 months than that in the chronic *Hyal2*−/− mice. Another measure of left ventricular (LV) diastolic function, the isovolumetric relaxation time, was significantly increased in all *Hyal2*−/− mice at all time points (Figure 2B). The reduced early to late atrial filling ratio and prolonged isovolumic relaxation time show there is an increased interval between mitral valve closure and aortic valve opening, indicating severe diastolic dysfunction in both acute and chronic *Hyal2*−/− mice compared with controls, although the acute *Hyal2*−/− mice were severely affected at earlier ages.

To evaluate systolic function, the ejection fraction and fractional shortening of acute and chronic groups of *Hyal2*−/− mice and controls were compared. No significant difference was observed among the groups (Figure 2C and 2D). LV corrected mass was assessed to determine whether hypertrophy detected by histological studies in *Hyal2*−/− mice was present at the level of the whole heart. Consistent with these earlier findings, both acute and chronic groups of *Hyal2*−/− mice showed progressively increased LV mass compared with control mice (Figure 2E). In the first 12 weeks, acute *Hyal2*−/− mice had reduced LV mass compared with control and chronic *Hyal2*−/− mice (Figure 2E).

Global cardiac function, represented by the myocardial performance index, was impaired in all *Hyal2*−/− mice. This value was significantly increased at all time points, reflecting the reduced overall cardiac performance (Figure 2F). Reduced cardiac output was also evident in the acute *Hyal2*−/− mice compared with chronic *Hyal2*−/− mice and controls (Figure 2G). Together, our data suggest that severe diastolic dysfunction accompanied by reduced cardiac output contributes to the development of heart failure in the acute *Hyal2*−/− mice within the first 3 months of life. In the chronic *Hyal2*−/− mice, progressive diastolic dysfunction without reduced cardiac output developed over time, leading to heart failure at an average age of 6 months.

**Increased Tissue Density in *Hyal2*−/− Mice**

Histological analyses of transverse sections of hearts from *Hyal2*−/− and control mice were used to investigate the basis of the increased tissue density. Hematoxylin and eosin staining revealed enlarged atria in all *Hyal2*−/− mice compared with controls, consistent with the ultrasound findings (Figure 3A through 3C, n=7 pairs). Further, in atria from the acute *Hyal2*−/− mice, tissue masses (*) were present that were absent in chronic *Hyal2*−/− and control mice (Figure 3A, n=4 pairs). HABP staining to detect HA revealed abundant HA in the atrium and ventricle of acute and chronic groups of *Hyal2*−/− mice compared with controls (Figure 3D through 3F). However, increased HA was only detected in the periphery of the atrial masses and not in the central region, which seemed to be composed of cardiomyocytes (Figure 3D, open arrow). Additionally, HABP staining revealed valve-like tissue in
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other regions of the heart of both acute and chronic Hyal2−/− mice compared with controls (arrow in Figure 3E). Masson trichrome staining demonstrated fibrosis in the atrium and ventricle of Hyal2−/− mice (acute and chronic, green arrows) compared with controls (Figure 3G through 3I; Figure III in the Data Supplement).

The excess fibrous tissue suggested there may be large numbers of fibroblasts secreting ECM. Indeed, an abundance of cells positive for the mesenchymal marker, vimentin, were detected. In a representative image of the atrium, excess vimentin-positive cells were obvious in the Hyal2−/− mice compared with controls, although the number of mesenchymal cells was

Figure 3. Histological analysis of hyaluronidase 2 (HYAL2)–deficient (Hyal2−/−) and control hearts. Transverse sections of hearts from Hyal2−/− (acute and chronic) and control mice were compared for differences in morphology and structure. A–C, Images of hematoxylin and eosin–stained sections revealed an enlarged atrium (arrow) in both the acute (A) and chronic (B) groups of Hyal2−/− mice compared with control mice (C). *A tissue mass in the atrium of the acute Hyal2−/− mouse. D–F, Images of hyaluronan (HA) distribution in the Hyal2−/− and control hearts. HA was detected as a brown precipitate using the HABP (HA-binding protein). There is intense brown staining in several regions of the Hyal2−/− hearts (open arrows in D and E), whereas the intense brown staining is limited to the valves in the control hearts (open arrow in F). G–I, Masson trichrome staining of Hyal2−/− and control hearts. Masson trichrome stains the extracellular matrix (ECM) components collagen and elastin as blue, and glycosaminoglycans remain unstained. Excess ECM indicating fibrosis (green arrows) is widespread in the Hyal2−/− hearts compared with the control heart (I). G′–I′, Enlarged view of the area in the box in (G)–(I). J–L, Detection of mesenchymal cells in Hyal2−/− and control hearts. Anti-vimentin (brown) indicates the presence of mesenchymal cells. There are increased numbers of vimentin-positive cells (arrow) in both the acute and chronic Hyal2−/− atria (J and K) compared with the control atrium (L). M, Semi-quantitative analysis of vimentin-positive cells in Hyal2−/− and control atria. N and O, Vimentin protein levels in Hyal2−/− and controls hearts (atrium and ventricle). N, Western blot analysis showed increased expression of vimentin in the Hyal2−/− heart compared with controls. GAPDH was used as protein-loading control. O, Quantification of vimentin levels in (N). The chemiluminescent images from Western blot analysis of vimentin from Hyal2−/− and control hearts (n=4) were quantified using a BioRad ChemiDoc. The columns represent the average level of vimentin±SEM (n=4). Significance was determined using the Student t test. Bar=50 μm. The images in this figure are representative of those from 7 pairs of Hyal2−/− and control mice.
significantly higher in the acute compared with the chronic *Hyal2*/*−* mice (Figure 3J through 3M). Similarly, mesenchymal cells in the ventricle were also higher in the *Hyal2*/*−* mice compared with controls (Figure III in the Data Supplement), although in this case, the number of vimentin-positive cells was higher in the chronic than in the acute *Hyal2*/*−* and control mice (Figure IIIIM in the Data Supplement). The increase in the level of vimentin in *Hyal2*/*−* mice was also detected using Western blot analysis of whole hearts (Figure 3N and 3O).

**Morphological Analysis of Embryonic Heart in *Hyal2*/*−* and Control Mice**

To determine whether the structural abnormalities in the adult heart of the *Hyal2*/*−* mice originated during development, we analyzed *Hyal2*/*−* and control hearts at E18.5, after the 4-chambered heart had formed. Hematoxylin and eosin staining revealed an enlarged atrium (Figure 4A, open arrow) and excess fibrous tissues in *Hyal2*/*−* mice (arrow in Figure 4A, n=8) compared with controls (Figure 4B, n=5). Further, HABP confirmed the presence of excess HA in the *Hyal2*/*−* atrium compared with controls (Figure 4C and 4D, n=3). The excess fibrous tissue was also accompanied by significantly increased numbers of vimentin-positive cells in the atria and ventricles of *Hyal2*/*−* embryos compared with controls (Figure 4E through 4H, n=3 and 4).

**Abnormal EMT in *Hyal2*/*−* Mice**

Previous ex vivo studies at E9.5 showed that high-molecular-mass HA promoted EMT, whereas HA fragments inhibited EMT and activated the VEGF pathway. To study this in vivo, we first analyzed the distribution of HYAL2 and HA in embryonic tissues of wild-type mice at E8.5, 11.5, and 12.5. HYAL2 was detected primarily in the endocardial lining of the developing E8.5 heart (Figure 5A through 5F), the bulbus cordis (open arrow), endocardial cushion, and wall of the ventricular chamber (arrow) of the developing E11.5 and E12.5 heart (Figure IV in the Data Supplement). The specificity of the HYAL2 signal was verified by comparing the pattern in wild-type and *Hyal2*/*−* E14.5 and E18.5 embryos (Figure 5G through 5J). HA was most abundant in the E8.5 developing heart, and progressively decreased during development (Figure IV in the Data Supplement). These findings are consistent with HYAL2 having a role in the degradation of HA during development.

Examination of the hearts at E14.5 showed the presence of fibrous tissues and HA in the atrium and ventricle in *Hyal2*/*−* mice (n=3) compared with controls (n=3; Figure 6A through 6D). Consistent with increased EMT in the *Hyal2*/*−* heart, there were significantly increased numbers of vimentin-positive cells (Figure 6E, 6F, 6I, and 6J) and decreased levels of VEGF (Figure 6G and 6H). Therefore, our data suggest that disruption of normal HA catabolism in the heart results in increased EMT. However, it is possible that the increase in vimentin-positive cells is because of increased mesenchymal cell proliferation alone or in combination with increased EMT.

**Discussion**

HA is abundant in the provisional matrix of the developing embryo. Its importance in heart development was clearly demonstrated by the early embryonic death of mice deficient in HA synthesis (HAS2 deficient). Without HA, EMT in the cardiac cushions of *Has2*/*−* embryos was not supported to form the heart valves and septum. Previously, we have demonstrated that a failure to degrade HA also resulted in
cardiac abnormalities in Hyal2−/− mice and HYAL2-deficient humans.5,6 Herein, we show that these abnormalities, and others, are present by 4 weeks of age and result in progressive and severe diastolic dysfunction. The persistence of HA in the absence of HY AL2 presumably promotes EMT and mesenchymal cell proliferation, resulting in excess mesenchymal cells in all Hyal2−/− mice, providing a molecular explanation for the fibrosis, and abnormal heart structures including thickened valves and atrial masses.

A specific role for hyaluronidase in development has been proposed in studies of the cardiac cushion and muscles of the embryonic chick23 and by in vitro studies showing opposing roles for high- and low-molecular-mass HA in EMT using cardiac cushion explants.11 Our studies provide in vivo evidence for the role of HYAL2 in EMT-related cardiac abnormalities in Hyal2−/− mice.

Figure 5. Hyaluronidase 2 (HYAL2) distribution in embryonic hearts. Sections of the embryonic heart at embryonic day (E) 8.5, from a previous study,19 were used for the detection of HYAL2 using immunofluorescent and immunohistochemical approaches. A–C, Detection of HYAL2 (red) in the endocardial lining of the blood vessels of the E8.5 heart. Nuclei are stained blue with Hoechst 33342. D–F, Enlarged view of the image in (A)–(C). G–J, HYAL2 distribution in E14.5 and E18.5 embryos. The brown staining indicates the presence of HYAL2 in the endothelial cells of blood vessels and heart valves of wild-type heart at E14.5 (H; n=3) and E18.5 (J; n=3), respectively. As expected, this signal is absent in the Hyal2−/− hearts (G and I; n=3). Bar=50 μm.

Figure 6. Histological analysis of Hyal2−/− and control hearts at embryonic day (E) 14.5. A–B, Images of hematoxylin and eosin-stained sections show excess tissue in the ventricle in hyaluronidase 2 (HYAL2)–deficient (Hyal2−/−; A) compared with control (B) hearts. C and D, Detection of hyaluronan (HA; brown) showed increased HA in the Hyal2−/− ventricle (C) compared with the control (D). E and F, Vimentin-positive cells were found to be more abundant in the Hyal2−/− heart (E) compared with the control (F). G and H, VEGF (brown) seemed to be more abundant in the Hyal2−/− (G) than in control (H) heart. I and J, Semi-quantitative analysis of vimentin-positive cells in the atrium (I) and ventricle (J) of Hyal2−/− and control hearts at E14.5. The number of mesenchymal cells in the Hyal2−/− hearts was significantly increased compared with controls. Bar=50 μm. **P<0.001, n=3; LA indicates left atrium; and V, ventricle.
that normal cardiac development in the mouse requires the hyaluronidase, HYAL2. The absence of HYAL2 results in the accumulation of high-molecular-mass HA4 and excess mesenchymal cells. Taken together, this suggests that HYAL2 is normally required to remove HA to inhibit EMT and mesenchymal cell proliferation and promote differentiation. Although we focused on the characterization of the cardiovascular defect in Hyal2−/− mice, craniofacial abnormalities and a missing kidney also affect a proportion of Hyal2−/− mice.5 HA may also be important in the development of these organs because HA levels are elevated during embryonic development of these tissues.22 The early lethality of HAS2-deficient embryos prevented determination of whether HA was required for the development of organs other than the heart.

Taken together with previous studies of HAS2 deficiency, it is clear that HA levels must be regulated for normal heart development. Heart defects in HYAL2-deficient mice and humans show that increased HA poses a risk for abnormal heart development. Similarly, embryonic lethality because of abnormal heart development in HAS2 deficiency indicates that too little HA also disturbs development.3 In humans, a single case of partial HAS2 deficiency was associated with a ventricular septal defect24; a complete HAS2 deficiency is unlikely to be compatible with life. We have recently described humans with HYAL2 deficiency and demonstrated that the cardiac phenotypes, which included cor triatriatum, atrial enlargement, valvular thickening and accessory tissue, and dilated coronary sinus,6 were similar to those in the Hyal2−/− mice. In addition, both the humans and mice shared palatal abnormalities and hearing loss. Taken together, the Hyal2−/− mice provide an excellent model for further study of HYAL2 function and potentially for the development of therapies for the human disorder.

The cardiac abnormalities found in Hyal2−/− mice are uncommon in humans, although there are examples of excess EMT leading to valve thickening. For example, mutations in PTPN11 encoding the protein tyrosine phosphatase SHP2 result in valve thickening in Noonan syndrome.8 In mice deficient in ephrin-A1, aortic and mitral valves are thickened, and dilated coronary sinus,6 were similar to those in the Hyal2−/− mice. In addition, both the humans and mice shared palate abnormalities and hearing loss. Taken together, the Hyal2−/− mice provide an excellent model for further study of HYAL2 function and potentially for the development of therapies for the human disorder.

In this and a previous study,3 Hyal2−/− mice clearly fell into 2 groups differing in the severity of their heart phenotype. In the previous study, the acute group exhibited severe atrial dilation leading to death at ≈ 3.2 months of age, whereas the chronic (nonacute) group died at ≈ 5.8 months of age.2 In this study, the acutely affected mice died earlier than in our previous study, and often this occurred soon after an ultrasound evaluation, suggesting that the anesthesia may have worsened the cardiac function. Ultrasound evaluation revealed that atrial dilation, valve hypertrophy, and diastolic dysfunction were already present at 4 weeks of age. Although overall cardiac function decreased until death, the structural parameters did not change significantly. The excess tissue growth found in the hearts of the acute mice was consistent with the finding of increased numbers of mesenchymal cells and ECM that results in rapid-onset diastolic dysfunction. Our findings are consistent with several studies that showed that in the presence of preserved systolic function the increased isovolumic relaxation time and atrial size27 are indicators of diastolic dysfunction, that are independent of atrial pressure,28 heart failure,29 or heart rate.30 The basis for the differences in the severity of the phenotype in acute and chronic groups of Hyal2−/− mice is probably because of a genetic determinant segregating in the outbred background. Modifying genes that influence the severity of a cardiac phenotype are extremely common,11 and future studies are required to determine the modifying genes that are involved in the acute and chronic phenotypes of the Hyal2−/− mice.

Thickening of the heart valves and walls and restricted blood flow and regurgitation through the affected valves (data not shown) were present in all Hyal2−/− mice. Together with abnormally placed valve tissues, these phenotypes are the probable cause of diastolic dysfunction. Several studies show that LV hypertrophy,23 interstitial fibrosis,35 and thickened valves34 are the principle causes of diastolic dysfunction of the heart. In the acute Hyal2−/− mice, the presence of tissue masses in the atria further disrupted cardiac function, resulting in earlier and more severe diastolic dysfunction and heart failure. In the chronic Hyal2−/− mice, the increased fibrosis in the ventricles may have resulted from compensatory changes for the ongoing diastolic dysfunction in the heart. In both cases, the eventual outcome was cardiac failure, although it is interesting that the ejection fraction was preserved in the chronic Hyal2−/− mice as models of this type are rare.

The impact of interstitial fibrosis on cardiac function is seen in other disorders of ECM molecules. Normally, the ECM provides support for the contractile forces produced by the cardiac myocytes, and disruption of ECM homeostasis can result in impaired force transmission, causing dilation or hypertrophy. For example, in ADAMTS 9- or 5-deficient mice, accumulation of versican in the heart disrupts ECM homeostasis and causes cardiac disease pathology.35,36 In addition, accumulation of glycosaminoglycans in the heart valves resulted in valve thickening that changed the atrial and ventricular volume overload and contributed to atrial dilation, ventricular hypertrophy, and ultimately diastolic dysfunction in many mucopolysaccharidoses.35 The cardiac disease pathology appears early in the life of patients with defects in glycosaminoglycan degradation and progresses rapidly to cause heart failure and sudden death. Almost 60% to 90% of patients with mucopolysaccharidoses have valvular disease.38 Surgical replacement of heart valves and continuous monitoring of cardiac function through echocardiogram are the common practice in the treatment of mucopolysaccharidoses patients with cardiovascular disease. Given that the valve thickening in our model occurs postnatally, generating a model with a postnatal deletion of HYAL2 might be beneficial for the study of valve disease.

These studies of the cardiac phenotype in Hyal2−/− mice clearly demonstrate an important role for HYAL2 and HA degradation in heart development. The presence of increased numbers of mesenchymal cells and decreased VEGF expression in Hyal2−/− embryos strongly suggests that HYAL2 is needed to inhibit EMT and that in its absence excess EMT leads to congenital malformations. Further studies are needed to clearly differentiate the effects of HYAL2 deficiency on
EMT and mesenchymal cell proliferation and to determine whether it is the presence of excess high-molecular-mass HA or the absence of low-molecular-mass HA that results in the phenotypic changes.

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Disclosures
None.

References


CLINICAL PERSPECTIVE

Hyaluronan is an abundant component of extracellular matrix that has been shown in mice to be required for normal cardiac development. As well, human cardiac pathologies such as cardiac fibrosis and myxomatous valve degeneration are associated with increased levels of hyaluronan. Recently, we have identified mutations in the hyaluronan-degrading enzyme, hyaluronidase 2 (HYAL2), as the cause of an autosomal recessive syndrome characterized by severe cardiovascular and palatal abnormalities. Comparison of humans and mice with HYAL2 deficiency revealed that several of the cardiovascular abnormalities, including thickened heart valves, enlarged atria, and cor triatriatum sinister, were shared. In the current article, we examine the origin and functional impact of the cardiovascular abnormalities in HYAL2-deficient mice. We found abnormally distributed valve-like tissue in the atria and ventricles, cell masses in the atria, and increased levels of hyaluronan and mesenchymal cells throughout the heart of HYAL2-deficient mice. These findings were associated with early-onset diastolic dysfunction with preserved ejection fraction that ultimately progressed to systolic heart failure in the mice. These studies clearly demonstrate an important role for hyaluronan degradation in normal heart development and function. Individuals with HYAL2 deficiency typically have cardiac anomalies and may be at risk for the development of heart dysfunction with age. HYAL2-deficient mice could be a valuable tool to determine the risk for further cardiovascular complications because of HYAL2 deficiency and for testing therapeutic interventions for these conditions.
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**SUPPLEMENTAL MATERIAL**

**Figure S1.** Valve thickening in *Hyal2−/−* and control mice. B-mode image of high frequency ultrasound exhibiting thickened aortic and mitral valves in the *Hyal2−/−* mouse (arrow, A,C) compared to the control (arrow, B, D). This is a representative image from n=14 pairs of mice.

**Figure S2.** E/A ratio in *Hyal2−/−* and control mice. M-mode image of mitral early (E) and late atrial (A) flow was calculated to evaluate the E/A ratio of *Hyal2−/−* and controls. *Hyal2−/−* (A) showed reduced E and increased A in comparison to that of the control mouse (B).
Figure S3. Histological analysis of the ventricle of Hyal2<sup>−/−</sup> and control hearts. Transverse sections of hearts from Hyal2<sup>−/−</sup> (acute and chronic) and control mice were stained for different cellular components. (A-C) H & E staining of the heart revealed fibrous tissues in the ventricle region of the acute (A) and chronic (B) Hyal2<sup>−/−</sup> mice compared to control mice (C). (D-F) Detection of HA in Hyal2<sup>−/−</sup> and control ventricles using the HABP. HA (brown) was abundant in the ventricle of the Hyal2<sup>−/−</sup> acute and chronic groups (D-E) compared to the control (F). (G-I) Masson’s trichrome staining of the ventricle of Hyal2<sup>−/−</sup> and control mice. The atrium of Hyal2<sup>−/−</sup> (G,H) show increased collagen (light blue) and accumulation of GAGs (white) compared to
controls (I). (J-L) Detection of mesenchymal cells in Hyal2−/− and control heart. Vimentin was detected using anti-vimentin (ab45939) and detected as a brown colour. Increased numbers of vimentin positive (brown color) cells are evident in the Hyal2−/− heart (J,K) compared to control (L). Scale bar = 50 µm. † \( P<0.05; ** \ P<0.001. \)

**Figure S4:** HYAL2 and HA distribution in wild type embryos. Sections of the embryonic heart at E8.5, 11.5 and 12.5 from a previous study\(^1\) were used for the detection of HYAL2 and HA using histochemical approaches. (A) HYAL2 (brown) in the wall of the bulbus cordis (open arrow) and endocardial lining of the truncal region (arrow) of the E8.5 heart. (E) HYAL2 in the endocardial cushions (arrow) of the outflow tract and the trabeculated wall of the ventricular chamber of the E11.5 heart. (H) HYAL2 in the endocardial cushions and the aortic-pulmonary spiral septum at E12.5. (A,D,G) HYAL2 was not detected in sections where no primary antibody
was used. (C, F, I) HABP staining in the heart showed a progressive decrease in HA with increasing embryo age.