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Short communication

Reduced aggrecan expression affects cardiac outflow tract development in zebrafish and is associated with bicuspid aortic valve disease in humans

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A B S T R A C T

Hemodynamic forces have been known for a long time to regulate cardiogenic processes such as cardiac valve development. During embryonic development in vertebrates, the outflow tract (OFT) adjacent to the ventricle comes under increasing hemodynamic load as cardiogenesis proceeds. Consequently, extracellular matrix components are produced in this region as the cardiac cushions form which will eventually give rise to the aortic valves. The proteoglycan AGGRECAN is a key component of the aortic valves and is frequently found to be deregulated in a variety of aortic valve diseases. Here we demonstrate that aggrecan expression in the OFT of developing zebrafish embryos is hemodynamically dependent, a process presumably mediated by mechanosensitive channels. Furthermore, knockdown or knockout of aggrecan leads to failure of the OFT to develop resulting in stenosis. Based on these findings we analysed the expression of AGGRECAN in human bicuspid aortic valves (BAV). We found that in type 0 BAV there was a significant reduction in the expression of AGGRECAN. Our data indicate that aggrecan is required for OFT development and when its expression is reduced this is associated with BAV in humans.

1. Introduction

Arterial valve leaflets are composed of three distinct layers of Extracellular Matrix (ECM) called fibrosa, spongiosa and ventricularis. The spongiosa layer is particularly rich in proteoglycans which will provide compressive properties to the tissue and allow the leaflet to change shape during the cardiac cycle. Indeed, valves are submitted to extreme hemodynamic forces such as shear stress and cyclic strain that will subsequently regulate both their development and function. Studies in chick and zebrafish have shown that disruption of the hemodynamic forces results in cardiac valves defects including defects of the outflow tract (OFT) cushion formation and severe heart defects associated with a total absence of valves [1,2]. Among valve diseases, bicuspid aortic valve (BAV) is one of the most common pathologies found in patients, occurring in 1–2% of living births and are frequently associated with aortic stenosis, regurgitation, endocarditis and calcified valves [3].

AGGRECAN is one of the major members of the large proteoglycans found in cartilage and provides the ability to resist compressive loads [4]. A recent transcriptomic study of human BAV has shown that AGGRECAN expression is decreased in BAV patients with mild calcification compared with calcified tricuspid aortic valve (TAV) [5]. Zebrafish possess 2 aggrecan paralogues, aggrecanA (acana) and aggrecanB (acanb). Here we show that acana expression in the zebrafish OFT is dependent on hemodynamic forces and that knockdown or knockout of acana during development induces cardiovascular defects. Moreover, we observe that in humans, AGGREGAN (ACAN) expression is reduced in type 0 BAV compared to normal TAV.

2. Materials and methods

2.1. Zebrafish strains and husbandry

Zebrafish were maintained under standardized conditions [6] and experiments were conducted in accordance with the European Communities council directive 2010/63. The Tg(fli1a:GFP)y1 line was provided by the CMR.[8]

2.2. Ruthenium Red treatment

At 3dpf, larvae were incubated in a 10 or 20 µM Ruthenium Red (RR) solution (Sigma, R2751) in E3 medium during 24 h.
2.3. In situ hybridisation

ISH were performed as described previously [7,8] (see Supplementary information). For RR treated fish, the proteinase K treatment was reduced to 15 min.

2.4. Morpholinos and injections

Morpholino oligonucleotides were obtained from Gene Tools (Philomath, OR, USA) and injected into one-cell stage embryos (see Supplementary information).

2.5. CRISPR/Cas9

Acana target sequences were identified using ZiFiT online software [9]. 150 pg of acana gRNA was co-injected with nls-Cas9 protein (NEB) (see Supplementary information).

2.6. Cardiovascular parameters analysis

Cardiovascular parameters were determined using the MicroZebraLab™ software from ViewPoint [10] (see Supplementary information).

2.7. Real-time quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Human aortic valve tissues were collected after surgery by the Department of cardiac surgery at "La Timone" Hospital, Marseille, France. The protocol was evaluated and authorised by the "CPP Sud Méditerranée" n°: 13.061. and by the "Agence de la biomedicine" n°PFS14-011. (see Supplementary information).

3. Results

3.1. AggrecanA is expressed in the zebrafish OFT and is dependent on hemodynamic forces

To determine the expression pattern of aggrecan, we performed in situ hybridization (ISH) on 4 days post-fertilization (dpf) embryos using antisense acana and acanb probes. Acana showed a high expression in craniofacial cartilage as previously described [11], however we were also able to observe a clear expression of acana in the OFT (Fig. 1.A). In contrast, we could not detect any acanb expression in this cardiac structure (Fig. 1.B). In order to confirm that acana expression was localised to the OFT, we performed double fluorescent ISH on 4dpf larvae using antisense probes targeting acana and elastin b (elnb), an abundant component of the zebrafish OFT [12]. In this manner we were able to observe that acana expression co-localised with elnb expression in the OFT (Fig. 1.C–H). Because ECM composition can be modulated by hemodynamic forces, we sought to determine whether this was the case for acana expression in the OFT. To achieve this, we used a previously described morpholino targeting tntt2 which effectively stops the heart from beating [13]. In this manner we found that tntt2 morphants display an apparent lack of acana expression in the OFT (Fig. 1.I). Previous research has indicated that mechanosensitive ion channels (MSC) can detect hemodynamic forces and trigger valve formation [14]. To determine whether MSC could mediate acana expression in response to hemodynamic forces, we employed the nonselective MSC blocker Ruthenium Red (RR). In
this manner we could observe that larvae incubated with RR showed a
dose responsive decrease of acana expression in the OFT (Fig. 1.J–L).

3.2. AggrecanA is involved in cardiac development

Due to its expression in the OFT, we speculated that acana may play a
role in cardiac development. To answer this question we adopted a
morpholino (MO) mediated approach targeting an internal splice site.
Injection of this MO produced a phenotype characterized by defective
cardiogenesis including a larger atrium and associated edema at 3dpf
(Fig. 1.M–N, P–Q). To confirm the specificity of the phenotype, we
performed several control experiments (Suppl. Fig. 1 and Suppl. informa-
tion). We also implemented a previously described transient CRISPR/
Cas9 knockout strategy [15,16]. In this manner we were able to observe
zebrafish embryos displaying a similar cardiac phenotype to that ob-
served when acana was knocked-down with a MO (Fig. 1.O,R). Al-
though this approach will result in a mosaic knockout of acana, all
embryos which displayed a cardiac phenotype tested positive for KO
of acana by T7 assay (n = 6/44) (Suppl. Fig. 2). Together these results
indicate that loss of acana either by knockdown or knockout leads to
perturbed cardiogenesis.

To better understand how the OFT has been affected, we analysed
this structure in Tg(fl11a:GFP)y1 larvae which express GFP in all endo-
thelial cells. At 3dpf, the wild-type OFT displays a typical “pear-shape
structure (Fig. 2.A). By contrast, in the acana morphants this structure
has failed to develop (Fig. 2.B). In parallel, we also analysed a number
of cardiovascular parameters such as heart rate and stroke volume. No
significant differences were observed in the heart rate between the
wild-type and acana morphants (Suppl. Fig. 3). However, the cardiac
output and stroke volume were significantly decreased in acana
morphants when compared to controls (Fig. 2.C and Suppl. Fig. 3). We
also recorded high-speed movies of the beating embryonic heart and
observed that in acana morphants there was a significant amount of
blood regurgitation between the ventricle and the atrium, most likely
caused by failed OFT development, forcing blood back through the AV
canal (Suppl. movies M1 and M2).

3.3. ACAN expression is reduced in human BAV patients

Based on our findings in zebrafish, we hypothesised that reduced
AGGRECAN (ACAN) expression may also be associated with aortic valve
diseases such as BAV. Firstly, we determined the relative abundance of
ACAN in the aortic valve by qPCR and were able to detect ACAN expression
during foetal valve development in human (Suppl. Fig. 4). By 13 weeks of
gestation, the ACAN expression greatly increased and this expression was
even more abundant in adult valves. To assess the possibility that ACAN
expression could be reduced in BAV patients, we performed RT-qPCR
analysis using RNA extracted from aortic valves surgically removed
from patients diagnosed type 0 “pure” BAV. As a control we used RNA ex-
tracted from normal TAV. We observed a significant (16 fold) decrease in
the expression of ACAN in the type 0 BAV samples when compared to the
control TAV samples (Fig. 2.D).

4. Discussion

Here we have shown that hemodynamically dependent acana ex-
pression is required for OFT development in zebrafish. Moreover, we
showed that a MSC could be the sensor of these hemodynamic forces.
It has recently been shown that the Trpv4 MSC, a target of RR, is in-
olved in atrioventricular valve development in response to hemody-
namic forces [14]. However, because RR is non-specific, we cannot
determine the true identity of the MSC at this juncture. Because the
analogous region in humans will give rise to the aortic valves, we also
analysed ACAN expression during human aortic valve development
and in patients who suffer from type 0 BAV we found a significant re-
duction when compared to TAV.

Because of the rarity of this condition, we were only able to analyse
relatively few patients, however the difference was in excess of 16 fold.
It will therefore be necessary to expand this cohort to determine fully
the reduction in the expression of AGGRECAN associated with BAV
type 0. At present little is known about the genetic causes of BAV,
with only a handful of genes thus far identified and, as one can imagine,
there is even less known about what causes the different types of BAV.
Although we cannot categorically state that reduced AGGRECAN expres-
sion is the root cause of type 0 BAV, its association with this condition
does appear to be significant. Decreased AGGRECAN expression with
BAV type 0 may not be so surprising considering it is required to
strengthen and provide rigidity to the developing valves, and when
this is lost the developmental process will malfunction. Why AGGRECAN
expression is reduced in BAV type 0 remains unclear at this juncture, how-
over it is possible that defective hemodynamics or defective
mechanosensation of these forces during OFT development could be in-
volved with this condition.

Supplementary data to this article can be found online at https://doi.

Conflict of interest

The authors report no relationships that could be construed as a con-
flict of interest.

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