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Reviewer A

Abraham and Hibino review stem-cell based clinical trials for HLHS patients, and then describe pre-clinical work for heart tissue patches. Overall, the review is well written.

Comment: Although the authors describe "limitations of stem cell injections," the foundational science for many of these clinical trials for congenital heart disease is based on controversial data of bone marrow-derived cardiac stem cells, resident cardiac stem cells within the heart itself, or injecting non-stem cells into the heart (commentary by J.A. Epstein, JAMA Cardiology, 2018; J. Kaiser, Science, 2018). In my view, I suggest that this controversy be acknowledged in some form among the limitations for these clinical trials.

Reply: Thank you for this input—this is an important point that should be addressed. This controversy has been acknowledged in the introduction with the following text (pages 3-4, lines 52-57):

"It should be noted that there has been some controversy around the regenerative capacity of adult cardiac tissue, such that the original studies showing that bone marrow-derived pluripotent cells could integrate into cardiac tissue and drive regeneration have been called into question (9). However, the evidence for effective cardiac regeneration in pediatric settings is more robust, with both preclinical and clinical evidence that pediatric hearts can indeed remodel effectively due to endogenous pluripotent cells (10)."

Comment: With regards to tissue patches in Sugiura, Hibino et al. 2016, the suggestion that the seeded cells (iPSC-derived cardiomyocytes from Cellular Dynamics, not stem cells as noted?) had extravasated and promoted the growth of endogenous cells is certainly an intriguing possibility. A minor comment for this review would be to suggest that this possibility could be evaluated in the future by genetically marking the iPSC-CMs on the patch to determine if donor cells extravasate outside of the patch (perhaps by following a human-specific gene if these are human iPSC-derived CMs), or by genetically marking host tissue to determine if rat host cells migrate on the patch.

Reply: This is a great suggestion, and has been integrated into the text as follows (pages 10-11, lines 233-235):

"A useful follow-up study would be to genetically mark and track the fate of the seeded cells to identify where (if anywhere) they traffic to from the patch, in order to gain insight into the mechanism of patch re-population."

In general, I am in agreement with the revisions. However, I think that this sentence remains an overstatement, which is important to address.

However, the evidence for effective cardiac regeneration in pediatric settings is more robust, with both preclinical and clinical evidence that pediatric hearts can indeed remodel effectively due to endogenous pluripotent cells (10).

I would suggest tempering this statement, as I am not yet convinced that the published evidence should be considered "more robust" in a pediatric setting, nor does it "indeed remodel effectively due to endogenous pluripotent cells." In my view, the approach shows some promise in the pediatric setting, although the mechanisms remain unclear.

Reply: This statement has been softened as follows (page 4, lines 55-58)—
"However, there is evidence for effective cardiac regeneration in pediatric settings, with preclinical and clinical data that suggest that pediatric hearts can remodel due to endogenous pluripotent cells, though the mechanism for

this remains unelucidated (10)."

I think that the revisions (page 4, lines 55-58) remain an overstatement and do not sufficiently address my continued concerns. In my view, the approach shows some promise in the pediatric setting, although the mechanisms remain unclear.

Reviewer B

Comment: HLHS is an important congenital heart disease and the review illustrates brilliantly the clinical trials targeting it. Historically, this disease has been treated via a series of surgeries that allows the heart to use a single ventricle. 44 These surgeries are often a palliative measure, and heart transplantation is the only definitive therapy that remains for patients and their parents. Stem cell-based regenerative therapies could play an alternative strategic role for HLHS. The authors should implement this review considering the patients with associated minor and major developmental defects and the role of a heterotaxy, if this is occasionally associated with HLHS. Moreover, the role of pulmonary hypertension should be discussed, and how to prevent alteration in older patients.

Reply: Most of the trials that have been performed to date have excluded HLHS patients with significant associated developmental defects, to reduce confounding variables. Therefore, limited data exists about patients with multiple comorbidities, as noted in the following text (page 9, lines 204-207):

"Finally, it should be noted that the patient population is selected for HLHS patients who largely do not have other significant cardiac, hepatic, or renal comorbidities. This was presumably done to reduce confounding variables. Yet, this makes it difficult to generalize to those patients who have associated developmental disorders, such as those with heterotaxy, Turner syndrome, or Jacobsen syndrome (23)."

The role of pulmonary hypertension in these patients is an important point that has yet to be addressed by clinical trials, as none of them have had a sufficient duration of follow-up to evaluate whether stem cell-based therapy has any impact on the development of pulmonary hypertension. This gap in knowledge has been addressed with the following text (page 9, lines 192-194):

"In addition, further follow up needs to be done to determine whether these therapies have an impact on the downstream development of common complications such as pulmonary hypertension or protein-losing enteropathy."

Reviewer C

The authors present a brief narrative review on cardiac tissue engineering with stem cell-based regenerative therapies for the treatment of hypoplastic left heart syndrome (HLHS). They highlight trial evidence that, while still early, showed that the introduction of pluripotent cells into the heart may be safe, feasible, and capable of improving the right ventricular ejection fraction, and discuss the growing potential of stem-cell-based cardiac patches. I thank the authors for their work and have some comments to improve their manuscript:

Major Comments:

1. Introduction: can the authors provide data on the outcomes and prognoses of the management of HLHS? For example, the authors state that "these palliative measures sometimes fail," which is very vague for readers.

Reply: The introduction has been expanded to discuss this in more detail, with the following text (page 1, lines 34-48):

"Palliative surgery typically occurs in three stages. Stage 1 surgery is a Norwood procedure typically completed in the neonatal period that establishes systemic blood flow from the right ventricle (RV) to the aorta and shunts

blood to the pulmonary arteries. Stage 2 is partial cavo-pulmonary bypass in which the superior vena cava (SVC) is connected with the pulmonary arteries at 4-6 months of age. In Stage 3, the inferior vena cava (IVC) is connected with the pulmonary arteries at age 18 months-4 years, creating Fontan circulation (3,4).
Though the advent of these procedures has made survival possible at all, the long-term prognosis for these patients remains poor. One study of 244 HLHS patients born between 1998 and 2012 estimated that 63.5% of patients survived to 1 year of age, 58.6% to 5 years, 54.6% to 10 years, and 32.6% to 15 years (3). In some cases, patients are unable to tolerate one of the stages of the palliation process, and are reverted back to the prior stage and listed for heart transplant (5). It has been observed that outcomes for HLHS patient that require heart transplant are poor, with one study observing that 53% of these patients had survived at the 10 year time point (5). Patients who achieve full Fontan circulation often experience complications, including protein-losing enteropathy, arrhythmia, and pulmonary hypertension (6). Thus, there is a need to improve on the benefit conferred by surgical palliation procedures for HLHS."

2. Trials: can the authors elaborate on eligibility criteria, especially exclusion criteria, to help readers understand how generalizable findings may be?

Reply: This has been described for each of the completed trials that were discussed in page 5-8, lines 84-87, 98-100, 119-122, and 140-144

3. PERSEUS: the authors state that "As in the TICAP trial, none of the patients developed cardiac tumors." The relevance of cardiac tumors here is unclear without background information, although readers may know/assume this is related to concerns about the use of pluripotent cells. Can the authors clarify this to ensure all readers' understanding?

Reply: A paragraph discussing the theoretical risks of therapies using pluripotent cells has been added, with the following lines (page 4, lines 73-78):

"There are several safety concerns that are particular to pluripotent cells that needed to be addressed by the clinical trials. Because pluripotent cells maintain their reproductive functionality, there is the theoretical risk that they could start replicating in an uncontrolled manner and form tumors (16). Similarly, poor integration of new electrically conductive tissue into the existing structure has the potential to be a nidus for arrhythmia formation. Therefore, the clinical trials observed these particular adverse events as part of their safety profile."

4. Trials: the authors state that "There are also several studies that have been terminated or completed with no results posted." Can the authors comment on how many "several" is and why no information has been published or released on their results? There is a common publishing bias by which negative trials or, worse, trials with worrying adverse events are not published, whereas positive trials are published. Was this the reason here? Were there other reasons?

Reply: Discussion of this topic has been included. No reason was given for why these trials ended with no publication of results, and we do not wish to speculate. However, the existence of the trials has been acknowledged in both the text (page 8, lines 161-164) and the summary table.

"it is important to acknowledge the studies that have not come to fruition in addition to the positive studies in the field. There are two studies that have been terminated or completed with no results posted for reasons that have not been publicized. All identified studies are summarized in **Table 1**."

5. Ethics: can the authors comment on the ethics of cardiac tissue engineering with stem cell-based regenerative therapies?

Reply: Though this is an unquestionably important topic, it requires a more extensive discussion than is

within the scope of this particular review.

Minor Comments:

1. Trials: please provide the full names of the trials when introducing the abbreviated names.

Reply: This has been done in lines 79-80, 97-98, and 137-138

2. PERSEUS: can the authors clarify whether the statement "with 17 patients from each group making it to this end point" implies that the other 24 patients (58.5%) died during the three months follow-up, were lost-to-follow-up, or other? The current wording is confusing in light of the later statement that "In the end, 34 105 patients received the treatment, and were followed for 12 months."

Reply: Thank you for this comment, the language has been clarified in pages 5-6, lines 101-107 as follows:

"Heart function was evaluated at 3 months, before which 1 patient from the control group was excluded for an EF>60%, and 6 patients from the experimental group were excluded for EF>60% (n=3), infective endocarditis (n=1), and withdrawal of consent (n=2). In total, 17 patients from each group were included in the analysis. At the 3-month time point, the controls were given the option to receive the CDCs, and all of them elected to do this. In the end, 34 patients received the treatment (17 in the initial experimental group, and 17 in the control group that was allowed to cross over) and were followed for 12 months with evaluation of heart function via cardiac MRI."

Reviewer D

The Authors submitted their review on the stem-cell based clinical trials for Hypoplastic Left Heart Syndrome (HLHS) patients, and on the preclinical works for heart tissue patches.

Comment: First of all, as the Editor I was surprised by noting that among the Authors the name of Dr. Luca Vricella is missing.

As a colleague of Dr. Vricella from many years, I am fully aware that in his Department of Chicago Dr. Narutoshi Hibino is the responsible for the experimental research, and therefore his name is listed as the senior Author of this manuscript.

Nevertheless, as the invitation was sent to Dr. Vricella, I strongly suggest the Authors to include his name among the Authors, also because I am sure that he will be able to provide adequate input to the manuscript, particularly in relation to the potential clinical implications of these experimental studies.

Reply: Dr. Vricella has been given the opportunity to provide input and feedback and has been added to the author list accordingly.

Comment: With regard to the review, as the object has been divided in two parts, I suggest a complete separation between the stem-cell based clinical trials for HLHS and the preclinical works on heart tissue patches, in all aspects: presentation, current knowledge, limits, etc.

Reply: The paper has divided the two topics—one section entitled "Clinical Trials for Stem Cell-based Therapies for HLHS" and one section entitled "Preclinical Studies of Cellular Therapies for Cardiac Regeneration"

Comment: The Reviewers have already provided very useful suggestions to further improve the quality of the manuscript, and the Authors should consider providing a revised version, taking in account their comments.

In addition, for the part related to the stem-cell based clinical trials for HLHS the Authors should provide all data available relative to the following issues:

- difference among the various types of stem-cell available, with advantages and disadvantages, and the outcomes obtained in the experimental and clinical trials.

- importance of the choice among the various modalities of administration of the stem-cells, in particular related to the timeframe (first palliative surgical procedure or second stage), before, during or after cardiopulmonary bypass, and the way of administration (cardiopulmonary bypass machine, ascending aorta, coronary arteries), dosage of administration etc.

Reply: Information about the stem cell therapies used in the relevant clinical trials has been provided in the following text (page 4, lines 64-72):
"These cellular sources are different in terms of ease of accessibility and constituent cell populations. The generation of "cardiosphere-derived cells" (CDCs) requires intraoperative sampling of cardiac tissue, and subsequent expansion of stem cell-like (CD90+CD105+vimentin+) (12,13) cardiac cells. In contrast, umbilical cord blood-based therapy requires a prenatal diagnosis of HLHS but is more readily accessible than cardiac tissue at the time of birth. The umbilical cord blood is enriched for mononuclear cells, a heterogeneous population that includes stem cells (14). Bone marrow-derived mesenchymal cells are by far the easiest to acquire, especially as they can come from an allogeneic source and be used "off the shelf" in the case of products like Lomecel-B (15)."
A discussion of the various routes/timing of administration and cell types, as well as a comparison of the efficacy of the various cell types can be found here (page 8, lines 170-181):
"One purpose of these various trials is to establish which methodology is optimal for this application. There are two main points of variation between the trials: cell source and method of delivery. Between cardiac derived cells, bone marrow derived cells, and umbilical cord blood cells, the CDCs are the only ones that have clearly demonstrated improvement in RVEF, while others have demonstrated only stabilization/lack of decline to date. However, this analysis is complicated by the fact that the trials that used CDCs were also the only trials that administered the cells via catheterization 4-5 weeks after completion of palliation surgeries, while the UCB-MNC (NCT01883076) and MSCs (ELPIS) were administered intramyocardially during the second stage surgery. This makes it difficult to parse whether the efficacy observed in the TICAP and PERSEUS trials is primarily driven by the cellular source or the route. Notably, in the PERSEUS trial it was observed that the second group of patients that received the CDCs "late" had diminished improvement relative to those that got it "early"—suggesting that the timing of the cell administration relative to the palliation surgery does have some impact (18)."

This type of information not only will further enhance the quality of the manuscript, but will provide very useful knowledge to all readers interested in this field.'
In any case, the Authors have to be congratulated for all their efforts in pushing the boundaries of the knowledge in this matter, with the purpose of ameliorate the clinical future of this group of patients, currently with a grim expectancy and quality of life.

Again, we would like to thank the reviewers for their contributions to this manuscript.