Can the proliferative ability of chicken cardiomyocytes be assessed using flow cytometry?

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The study of the formation of new cardiac muscle cells during postnatal development is a relatively new field. During fetal development, new cells are formed as the heart grows. However, the proliferative ability of postnatal cardiomyocytes is still debated. While several studies have been made on mammals, less is known about the chicken cardiac cells and their postnatal proliferation. As almost all previous studies have used microscopy-based cell counting methods, there has been some limitations on accuracy and amounts of cells that could be counted. The aim of this study is to develop a method for using flow cytometry to analyze proliferative ability of chicken cardiomyocytes and to investigate if any postnatal proliferation exists. For this study, 4 weeks old Red Junglefowl (*Gallus gallus*) chickens were used for isolating cardiomyocytes. In addition, 19 days old Red Junglefowl embryos were used to assess if a longer incubation time would yield a higher number of proliferative cells. Cells were stained using a commercial EdU imaging kit and analyzed using flow cytometry and imaging flow cytometry. The produced results could not be used for determining the proliferative ability of the cardiomyocytes, but provides crucial information for possible method improvements. In conclusion, this study has laid important groundwork for future studies on the proliferative ability of chicken cardiomyocytes.

Flow cytometry, postnatal cell proliferation, cardiomyocytes, chicken, Red Junglefowl, *Gallus gallus*
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1 Abstract

The study of the formation of new cardiac muscle cells during postnatal development is a relatively new field. During fetal development, new cells are formed as the heart grows. However, the proliferative ability of postnatal cardiomyocytes is still debated and is highly species specific. While several studies have been made on mammals, less is known about the chicken cardiac cells and their postnatal proliferation. As almost all previous studies have used microscopy-based cell counting methods, there has been some limitations on accuracy and amounts of cells that could be counted. The aim of this study is to develop a method for using flow cytometry to analyze proliferative ability of chicken cardiomyocytes and to investigate if any postnatal proliferation exists. For this study, 4 weeks old Red Junglefowl (Gallus gallus) chickens were used for isolating cardiomyocytes. In addition, 19 days old Red Junglefowl embryos were used to assess if a longer incubation time would yield a higher number of proliferative cells. Cells were stained using a commercial EdU imaging kit and analyzed using flow cytometry and imaging flow cytometry. The obtained results could not be used for determining the proliferative ability of the cardiomyocytes, but provides crucial information for possible method improvements. In conclusion, this study has laid important groundwork for future studies on the proliferative ability of chicken cardiomyocytes.

2 Introduction

The heart is one of our most well-studied organs and new discoveries are still made. One of the main cell types in the heart are the cardiomyocytes, the muscle cells that cause the contractions we call heart beats. These cells are vital for the well-being of the heart and their reproductive ability is an interesting subject. In the neonatal mammalian heart, cells grow through two complementary mechanisms; hypertrophy (the increase in cell size) and hyperplasia (the increase in cell number) (Jonker et al. 2007). Earlier it was believed that the fetal cardiomyocytes went through terminal differentiation and lost their ability to proliferate before birth (Bugaisky and Zak 1979). However, newer studies have shown that the mammalian cardiac cells still undergo mitosis to some extent, for several days after birth (Li et al. 1996; Ali et al. 2014; Jonker et al. 2015). Some even claim that the cells retain their proliferative ability for several weeks or even years after birth (Mollova et al. 2013; Naqvi et al. 2014), but these studies have faced some critique (Alkass et al. 2015). While the duration of cardiomyocyte proliferation remains controversial, it is clear that the cells continue to divide even after birth.
While many studies have been made on different mammal species, other vertebrate models have not received the same attention. The chicken, which is a commonly used bird model, seem to have a similar proliferative ability as mammals (Li et al. 1997). However, the studies are sparse and leave much wanted in terms of methodology. Assessing the proliferation of chicken cardiomyocytes is therefore a novel field and could provide valuable input in the above mentioned discussion.

A common denominator for many of the previously conducted studies is that they are based on some type of microscopy observation method. Counting cells using these methods are oftentimes an arduous and time consuming task, with clear limitations on how many cells that can be counted. Since many of them rely on sight confirmation, human error and bias may become an issue. Newer methods have been developed to counteract these problems, but the issue with the amount of cells that can be counted remains (Benes and Lange 2001; von Bartheld 2002).

Another commonly used method for counting and studying single cells is flow cytometry, which recognizes and differentiate cells based on their morphology and fluorescent marking. By passing cells in a laminar flow through a laser beam, detectors can observe the different scatter patterns and fluorescence that the cells generate. These different attributes are used to sort cells into different subpopulations that can be easily quantified. While manual cell counting methods yield similar results in regards to cell fraction percentages, flow cytometry allows for significantly larger amounts of cells to be counted while maintaining a higher precision (Andersson et al. 1988; Bolanos et al. 1988; Collins et al. 2010). When paired with using thymidine analogue 5-ethynyl-2’-deoxyuridine (EdU), proliferative cells can be easily detected. If EdU is present during DNA-synthesis, it will incorporate into the DNA and a fluorochrome can later be attached to it to enable detection by the flow cytometer.

The goal of this study was to develop a method allowing proliferative chicken cardiomyocytes to be marked using EdU and then counted using a flow cytometer. The aim was to provide a more accurate and faster method of quantifying cell proliferation and to investigate if any cell proliferation exists in postnatal cardiomyocytes. As a proof-of-principle, differences in proliferation between cells incubated with EdU for different time durations has also been evaluated.
3 Material & methods

3.1 Animals and treatment

For this study, two 19 days old Red Junglefowl (Gallus gallus) embryo and one 4 weeks old Red Junglefowl chicken were used. The 4 weeks old animal was treated with EdU by injection into the ulnar vein (dosage at 4.7 µg/kg) approximately 24h before cell isolation. The embryos were treated by deposition of a small volume onto the inner cell membranes on the air cell after perforation of the eggshell with a thin drill (0.3 mm diameter) for 6h and 20h before cell isolation respectively. A 200 µl blood sample was taken from the ulnar vein of the 4 weeks old chicken before euthanasia.

3.2 Cell isolation

Cardiomyocytes were enzymatically isolated using retrograde perfusion according to a previously described protocol (Österman et al. 2015) with some changes. The animals were euthanized by decapitation and the chest cage was opened. 100 µl of a heparin solution (5000 U/ml) was injected into a major blood vessel of the heart to prevent clotting. The vessels returning blood from the body were tied off and then cut using a scissor, along with all remaining vessels. The heart was then removed from the chest cavity unto a petri dish with Tyrode’s solution (in mM: 140 NaCl, 5 KCl, 1 MgCl2, 10 glucose, 10 HEPES, adjusted with NaOH to pH 7.35) and a blunt needle was inserted into the aorta. The needle was tied in place and attached to a peristaltic pump (MINIPULS® 3, Gilson, Inc.). Coronary perfusion was initiated using Tyrode’s solution for approximately 15 min (10 min for embryos), until the heart had blanched. The perfusion fluid was changed to an enzymatic solution containing 160 U/ml type II collagenase (Worthington collagenase type 2, 230 U/mg) and 0.78 U/ml protease type XIV (protease from Streptomyces griseus, 3.5 U/mg, Sigma Aldrich) and perfused for another 20 min (15 min for embryos). Superfluous enzymes were flushed out using Tyrode’s solution for 15 min (10 min for embryos). All perfusion steps were done using a flow rate of 3.6 ml/min (0.9 ml/min for embryos) at a pressure of 2–3 kPa and perfusion solutions were kept at 39°C.
After complete perfusion, ventricles were separated from atria and outflow tract, and flushed out using a syringe to decrease blood cell content in the final sample. The ventricles were transferred to a Falcon tube containing Tyrode’s solution and the tube was shaken to mechanically loosen the cells. The solution was filtered into a new tube to remove incompletely dissolved tissue and then 100 mM CaCl$_2$ was added to the solution to a final concentration of 2 mM to promote cell contraction.

The solution was rested at room temperature for 15 min and then the cells were fixed by adding 2% Paraformaldehyde (PFA) to a final concentration of 1% PFA and incubated for 15 min. The PFA was removed and the cells resuspended in PBS.

3.3 Staining
Proliferative cells were labeled using a Click-iT EdU Alexa Fluor® 488 Imaging Kit (Invitrogen, Eugene, OR) with a modified protocol. Before staining, parts of the previously collected samples were transferred into Eppendorf tubes, while simultaneously filtered using a 50 µm Partec Celltrics filter. The PBS was removed and the Click-iT® EdU-staining cocktail was added. The cells were incubated in the dark for 30 min, then the cocktail was removed and the sample was washed twice using PBS and finally resuspended in PBS.

3.4 Flow cytometry
The proliferative ability of the cells was analyzed using a Gallios™ flow cytometer (Beckman Coulter, Inc). For every sample at least 5000 cells were recorded and the results were analyzed using Kaluza® Flow Analysis software (Beckman Coulter, Inc.). The cells were visualized using an ImageStream® Mark II Imaging Flow Cytometer (Amnis®).
4 Results

Scatter analysis of cells isolated from 4 weeks old chicken show large range in both forward scatter (dependent on cell size) and side scatter (dependent on cell granularity) (Fig. 1A-B) and show no clear groupings of cells. For the fluorescence graphs, the FL1-channel corresponds to the EdU fluorescence, while the FL7-channel only responds to background fluorescence. B+- quadrant represent EdU-positive cells and a majority (94.5%) of the stained analyzed cells were identified as EdU-positive (Fig. 1D) when compared to non-stained cells (Fig. 1C).

Figure 1. Dot plot of cells isolated from ventricles of a 4 week old Red Junglefowl chicken. Top panels show results from non-stained cells; bottom panels show corresponding results from cells stained with Click-iT EdU Alexa Fluor® 488 Imaging Kit. Samples show large scattering in both forward scatter (depending on size) and side scatter (dependent on the granularity of the cells) (A-B) and a majority (94.5%) are recorded as EdU-positive (D) when compared to non-stained cells (C).
Further analysis using ImageStream showed that a large portion of unstained cells are Red blood cells (Fig. 2A), indicating some blood contamination in samples. Cells recognized as EdU-positive (Fig. 2B) matches morphology of contracted cardiomyocytes in earlier observations in a light microscope (not shown).

Figure 2. Images of EdU-negative cells (A) and EdU-positive cells (B) from ImageStream Mark II Imaging Flow Cytometer. Non-positive cells are mostly identified as Red blood cells, indicating blood contamination in sample. Positive cells correspond to morphology of contracted cardiomyocytes previously observed using light microscopy. Red squares show typical Red blood cell (A) and cardiomyocyte (B).
A blood sample from the 4 weeks old chicken taken before cell isolation was analyzed similarly to the cardiomyocyte samples and showed a very large variation in regards to both forward scatter and side scatter (Fig. 3). This is an indication of a large size variety of the measured cells. The density of measured cells, along with the size difference was too high to allow for analysis of subpopulations.

Figure 3. Dot plot of blood sample from one 4 weeks old Red Junglefowl chicken, showing large variation in both side scatter and forwards scatter indicating a big size variety of measured cells. No subpopulations could be analyzed because of the large size variation and cell density of the sample.
Figure 4. Dot plot over samples from Red junglefowl embryonic hearts, incubated with EdU for 6 hours (A) and 20 hours (B) before euthanasia. Left column shows unstained cells and right column cells stained with Click-iT EdU Alexa Fluor® 488 Imaging Kit. A small difference could be detected between the two incubation times, 6h sample contained 37.5% positive cells (C, quadrant D++) and 20h sample contained 40.6% positive cells (D, quadrant B++) when compared to non-stained cell samples.

Lastly, a small difference could be seen between embryonic cells incubated with EdU for 6 hours (6h) versus 20 hours (20h). When compared to non-stained cells (Fig. 4A-B), 6h sample contained 37.5% EdU-positive cells (Fig. 4C) while the 20h sample contained 40.6% EdU-positive cells (Fig. 4D).
5 Discussion

The results of this study have proved that it is possible to evaluate chicken cardiomyocytes by using flow cytometry, but has not been able to clearly show if postnatal cardiac cells still possess reproductive abilities. Samples do not show enough separation between positive and negative populations to give clear answers about the proliferative ability of the cells. Below, I will suggest several improvements that can be made to increase the possibility of success.

When compared to values from previous studies, the high proliferation rates from the samples from the 4 weeks old chickens seem improbable. Li et al. (1997) found that cell number increased by around 1.7% per day between day 15 and 29. Since EdU is ingrained into all new DNA, both mother and daughter cell should be marked positive in the flow cytometric analysis. However, that would still only account to around 3.5% of EdU-positive cells when compared to the values Li et al. (1997) produced. Therefore, an EdU-positive population of around 95% is not probable. This is made even clearer when looking at results from postnatal sheep, where studies show that around 1% of cells still are active in the cell cycle 1 week after birth (Jonker et al. 2015). A more possible explanation of the high positive value would be that the sample contains too much background fluorescence to yield clear results. Too much background can have several explanations, but the two most common ones are that the sample contains a lot of debris and dead cells (which absorbs a large amount of dye and can cause false positives) or that the sample was treated with too much dye, which caused an excess of non-specific binding.

Since the scatter dot plot of the 4 weeks old chicken samples are similar to what others have obtained when analyzing cardiomyocytes (Bhattacharya et al. 2014) and that gating should remove debris particles from the fluorescence plots, the most probable explanations are excess amount of dead cells or the usage of too much dye. To account for dead cells, a viability stain could be used. Viability stains uses specific dyes that only stain live or dead cells and therefore make it simple to distinguish between true EdU-positive cells and dead cell false positives. Another improvement that can be done while staining cells is to stain cells before fixation. Lanier and Warner (1981) found that post-fixation staining significantly increases background fluorescence, so simply fixating after staining could improve the results. To determine if there is too much non-specific binding of the fluorochrome, a fluorochrome titration should be done. By analyzing a dilution series for the Alexa
Fluor® 488 azide used for staining, a suitable concentration of dye can be determined and non-specific binding can be reduced.

After analysis of the cells using the ImageStream flow cytometer, it was clear that the samples were contaminated with red blood cells. While the erythrocytes were not found in the EdU-positive population, the contamination still presents a problem since they cannot be distinguished from EdU-negative cardiomyocytes. To properly analyze the proportion of proliferative cells, EdU-positive cells should be presented as a fraction of the complete cardiomyocyte population. If there would be erythrocytes present in the samples, the fraction of proliferative cells may be found to be smaller than it is in reality and important results could be dismissed as non-significant. To prevent this, a blood sample from one of the 4 weeks old chickens was analyzed in a similar way as the cell samples. The idea was that if a clear population was seen, blood cells could be gated for in the same way as debris was. Unfortunately, the sample proved to contain too much debris and lysed cells to yield proper results. Should this approach be pursued further, blood samples should be washed and filtered properly for the complete removal of debris. Another approach for excluding red blood cells is specific labeling using antibodies. Antibody-labeling is a common approach when working with flow cytometry and often used when working with red blood cells (Wagner and Flegel 1998; Stroncek et al. 2003).

Compared to the 4 weeks old cell analysis, the embryonic cells indicate a lower proliferation rate where only up to 40% of measured cells were identified as EdU-positive. However, these numbers are still far higher than other studies have measured in near term embryonic cells (Marino et al. 1991; Soonpaa et al. 1996; Jonker et al. 2007). Similar to the results from the older cells, there is no real population separation within samples. Instead it seems as though all cells have become stained and that the positive cells are simply caused by the shift that the increased fluorescence creates. A slightly higher amount of EdU-positive cells was found in the 20h sample compared to the 6h, which could indicate that there are more proliferative cells. However, it is impossible to determine if it is a significant increase or only within the variation of measurements. Several additional individuals should be tested to rule out variation.

Much of the work done for this study was developing the method and one of the main issues was that the isolated cells remained elongated, which was a problem since rounds cells are necessary for flow cytometry analysis. Several different methods were tried, but were unsuccessful. Finally, a study on rat cardiomyocytes, where the authors had added 2 mM Ca$^{2+}$ to promote cell contraction was found (Armstrong and Ganote
and the method proved to be somewhat successful in obtaining cells that could be analyzed in the flow cytometer. However, since there still was other problems that had to be solved, no further optimization was done for this step because of the time limit. While the added calcium did produce round cells, some cells still kept their elongated shape (Appendix 1). It is therefore probable the results from the flow cytometry measurements could be improved if this step could be optimized properly.

Another problem that limited the reach of this study was that several of the samples could not be analyzed using the ImageStream flow cytometer, since the machine simply would not take them. It was concluded that this problem likely was caused by the fixation of the cells. The principle was that the Gallios flow cytometer processed the cells using a higher surrounding pressure compared to the ImageStream flow cytometer and that fixing the cells using paraformaldehyde negatively affected the cells by making them heavier. The conclusion was that the pressure of the ImageStream flow cytometer simply was not enough to handle the heavier cells, therefore making it impossible to process them. A solution for this would be to analyze the cells without fixing them beforehand. This could also be beneficial to the staining procedure, as brought up earlier.

5.1 Conclusion

This study has mainly focused on developing and improving a new method for studying cell proliferation in cardiomyocytes. While several problems were encountered during testing, the results have provided very valuable information for future testing and several improvements that could be made to the existing protocol. This study has laid the groundwork for future studies and if the suggested improvements are made, there is reason to believe that this method could yield satisfying results.

pers. comm., Jörgen Adolfsson, IKE/MMM, Linköpings universitet
5.2 Societal & ethical considerations

This study has aimed to develop a method to improve the work in studying the proliferative ability of postnatal cardiomyocytes. Further knowledge about the proliferative ability of postnatal cardiac cells and the mechanisms behind can be used as stepping stones for further research on the heart and its regenerative ability.

During the study, several animals have been euthanized to provide in vivo cell samples. Euthanasia have been achieved through decapitation, which is fast and does not affect the animal tissues. Decapitation causes no more distress to the animal than other methods of euthanasia. All procedures were approved by Linköpings Djurförsöksetiska Nämnd, the formally appointed district ethical committee under permissions Dnr. 9-13.

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7 References


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8 Appendix

Appendix 1. Photo of isolated cardiomyocytes from 4 weeks old chicken treated with 2 mM Ca$^{2+}$, showing both round and elongated cells.