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14. ABSTRACT

Epidemiologic evidence shows alteration of myocardial properties with age resulting in progressive decline in diastolic function. The presence of a population of senescent cardiomyocytes and the soluble factors they secrete (senescence-associated secretory phenotype) could be a major player in the progressive deterioration of myocardial function observed during aging. To test this hypothesis, we propose 1) to generate a new model of cardiac-specific inducible senescence and evaluate cardiac function in association with cellular senescence and 2) to develop a method for in vivo imaging of cellular senescence. The animal model of expected to develop cardiac-specific senescence was generated by crossing mice with cardiac specific inducible Cre recombinase (αMHC-MerCreMer) with enhancer of zeste homolog 2-floxed mice ($Ezh2^{fl/fl}$), enabling tissue-specific conditional deletion of Ezh2, a key component of a protein complex involved in the repression of $p16^{INK4a}$, a master regulator of cellular senescence. Tamoxifen treated double transgenic animals showed the presence of the recombined allele, marker of Ezh2 deletion, in heart tissue. The follow-up of the animals for up to 7 months after genomic recombination did not result in impaired cardiac function, as assessed by echocardiography. Histological evaluation did not show the presence of senescent cells in the heart tissue. The generation of malondialdehyde-vimentin, a marker reported to be expressed at the membrane of senescent cells, was unsuccessful. Murine cardiomyocytes were used to screen for alternative markers of cellular senescence. The absence of detectable cellular senescence in the generated animal model prevented the selection of a molecular target for tracer development with validated in vivo relevance for cardiac tissue, as initially anticipated.

15. SUBJECT TERMS

Cellular senescence, diastolic dysfunction, molecular imaging

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1. INTRODUCTION:

Indirect evidence suggest that cellular senescence could play a major role in age-related myocardial alteration leading to diastolic dysfunction. This work proposes to generate a murine model of cardiac specific inducible senescence, *via* tissue specific conditional knockout of enhanced of zeste homolog 2 (Ezh2), and evaluate the impact of cardiac specific cellular senescence on the development of diastolic dysfunction (Aim 1). Currently, there is no non-invasive imaging method to directly assess senescent cell burden in vivo. A second aspect of this work is to generate a tracer for molecular imaging of cellular senescence *in vivo* and validate its functionality and utility in the generated mouse model (Aim 2).

2. KEYWORDS:

Cellular senescence, diastolic dysfunction, molecular imaging

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Aim 1: Development of a preclinical model of cardiac-specific inducible senescence and evaluation of the relationship between cardiac cellular senescence and myocardial dysfunction.

Four key tasks were identified for Aim 1, covering the major tasks 1 and 2 presented in the statement of work: generation of homozygote double transgenic animals, test and optimization of genomic recombination, cross-sectional study, and validation study.

The generation of the mouse model of cardiac-specific and inducible senescence was expected to be achieved by crossing the cardiac-specific inducible Cre mouse (αMHC-MerCreMer), with whole body Ezh2-floxed mouse ($Ezh2^{fl/fl}$). The double transgenic line ($Ezh2^{fl/fl}$:αMHC-MerCreMer) was successfully generated.

The evaluation of the efficiency of genetic recombination showed detectable recombined allele with all the doses of tamoxifen tested.

Cross-sectional studies of $Ezh2^{fl/fl}$:αMHC-MerCreMer animals after genetic deletion of Ezh2 were carried out with up to 7 months of follow-up. No alteration of cardiac function was observed in the study animals, and partial evaluation of the collected tissue samples indicates no identifiable cellular senescence, contrary to the hypothesis driving this work.

The validation study was expected to be carried out at the optimal time point determined from the cross-sectional studies. With the negative results observed at the stage of the cross-sectional study, this part of the project designed to establish the correlation between cellular senescence and diastolic dysfunction could not be completed.

Aim 2: Development and validation of novel tracer for non-invasive imaging of cellular senescence.

Four key tasks were identified for Aim 2, covering the major tasks 3 and 4 presented in the statement of work, including: antigen preparation, immunization, phage-display and nanobodies production, radiolabeling and screening of tracer candidates and evaluation of the selected tracer. As presented in the previous progress report, the preparation of antigen identified from the literature: vimentin, modified by the oxidative adduct malondialdehyde (Proc Natl Acad Sci U S A. 2017, 114:E1668) was unsuccessful. As a result, the alternative strategy adopted consisted of the identification of alternative target using cardiomyocyte cell line. The potential targets that were identified were to be validated in the generated animal model. While initial steps of the validation were performed, absence of evidence of the desired phenotype in the generated animal model prevented further evaluation of their relevance. The alternative approach of phage-display on a peptide library, using senescent cardiomyocytes could not be completed.

What was accomplished under these goals?

Aim 1: Development of a preclinical model of cardiac-specific inducible senescence and evaluation of the relationship between cardiac cellular senescence and myocardial dysfunction.

The specific objectives were to generate homozygote double transgenic $Ezh2^{fl/fl}$:αMHC-MerCreMer animals, test and optimized the conditions of genomic recombination and performed validation studies ensuring that the expected phenotype was present; first using a cross-sectional design to identified the optimal time point for the study, and, in a second time, perform a more granular and more powered analysis at the selected time point.

Mice with loxP sites flanking exons 14-15 of enhancer of zeste homolog 2 gene ($Ezh2^{fl/fl}$) and mice with tamoxifen-inducible Cre recombinase under the promoter of cardiac-specific alpha-myosin heavy (αMHC-MerCreMer) were obtained from the Jackson Laboratories (JAX). Methods to identify the presence of transgene by PCR were developed based on the protocols described by JAX and validated in animals with known genotype. Single transgenic animals were crossed to obtain a first generation (F1) of double heterozygote animals ($Ezh2^{fl/+}$:αMHC-MerCreMer/wt). The heterozygotes $Ezh2^{fl/+}$;αMHC-MerCreMer/wt mice were crossed to obtained F2 animals, which were genotyped to assess the presence of transgenes. The double transgenic line ($Ezh2^{fl/fl}$:αMHC-MerCreMer) was successfully established by crossing F2 animals.

Several conditions for genomic recombination were tested using PCR on genomic DNA extracted from the heart of $Ezh2^{fl/fl}$:αMHC-MerCreMer mice. In all tested condition, genomic recombination was detected using primers specific of the sequence obtained after Cre-excision, while as expected, the wild type allele was also detected (Fig. 1). No excised allele was detected in tested non-cardiac tissue (liver) in all conditions. Quantitative approach to evaluate the degree of recombination from extracted genomic DNA did not reveal any difference between the conditions on average, although concern about the sensitivity of this methods remains. Additional tests for a quantitative and spatial assessment of genomic recombination using in situ hybridization to detect the recombined allele on tissue section were unsuccessful.

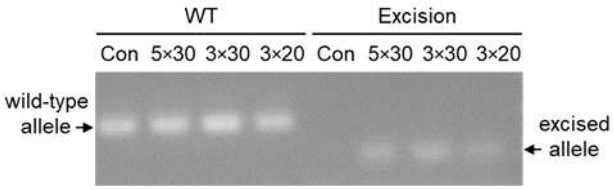

Fig. 1: Genomic recombination.
Detection of wild-type and Cre-excised allele in genomic DNA extracted from the heart of $Ezh2^{fl/fl}$;αMHC-MerCreMer mice after treatment with tamoxifen 3 × 20 mg/kg, 3 × 30 mg/kg, 5 × 30 mg/kg or vehicle solution.

Multiple groups of animals were treated with the initially proposed dose of tamoxifen based on the literature: 3 consecutive days of mg/kg tamoxifen administration (3 × 30 mg/kg), validated for its capacity to induce genetic recombination of the transgene, in order to evaluate progression of cardiac function over time in association with the cross-sectional evaluation of cardiomyocytes senescence. Evaluation of genomic recombination by quantitative PCR using double primer strategy did not provide additional insight into the extent of genomic recombination observed in the different experimental conditions.

Cardiac function was evaluated at baseline and followed-up for up to 6-7 months in $Ezh2^{fl/fl}$:αMHC-MerCreMer mice treated with tamoxifen 3 × 30 mg/kg or with vehicle solution. Table 1 presents the data from animals, in groups of animals pooled in 2-months post-treatment intervals, used to analyze with increased statistical power. For all animals, heart structure and function were evaluated prior to tamoxifen treatment and at a single of multiple time points post treatment. After correction for false discovery, no statistically significant difference was observed in any of the assessed parameters reflecting diastolic function between vehicle treated and tamoxifen treated animal group at any time point, nor between the different time points within the groups. Similarly, none of the structural characteristics of the heart or measures of systolic function was changed over time in either group or between groups at any studied time point.

High dose of tamoxifen 5 × 30 mg/kg resulted in acute mortality in the treated animal with 80% (8/10) of mortality at 3 days after the last dose. The high mortality was not observed in αMHC-MerCreMer animals suggesting that it resulted from the consequence of Ezh2 deletion in cardiomyocytes. Surviving animals at 3 days after treatment were euthanized for histological analysis. No echocardiographic evaluation of heart function was performed in animals treated with the high dose of tamoxifen.

	t_0		1 – 2 mo		3 – 4 mo		6 – 7 mo	
	Con (n = 19)	TAM (n = 41)	Con (n = 11)	Tam (n = 17)	Con (n = 15)	TAM (n = 19)	Con (n = 9)	TAM (n = 9)
Age (mo)	3.9 ± 1.0	4.1 ± 1.1	5.4 ± 0.7	5.1 ± 0.6	8.3 ± 1.1	8.1 ± 1.2	11.4 ± 0.7	11.3 ± 0.8
BW (g)	28.1 ± 5.4	29.4 ± 5.9	29.0 ± 3.9	28.8 ± 6.0	32.6 ± 5.5	32.8 ± 5.6	33.9 ± 6.0	34.8 ± 3.6
LV mass (mg)	95.7 ± 14.9	92.5 ± 16.3	97.2 ± 19.6	94.0 ± 13.1	98.2 ± 18.6	92.1 ± 12.8	111.3 ± 18.5	101.8 ± 17.9
LV mass/BW (mg/g)	3.51 ± 0.73	3.21 ± 0.54	3.33 ± 0.33	3.34 ± 0.48	3.04 ± 0.44	2.86 ± 0.49	3.31 ± 0.46	2.92 ± 0.38
LV vol.;s (µL)	21.2 ± 6.3	20.3 ± 6.4	20.0 ± 6.2	19.4 ± 6.2	26.7 ± 10.6	21.7 ± 8.1	21.1 ± 8.5	17.4 ± 8.1
LV vol.;d (µL)	68.7 ± 13.3	66.1 ± 9.3	67.4 ± 11.2	61.4 ± 6.6	75.2 ± 15.7	66.6 ± 8.5	72.9 ± 15.8	62.6 ± 8.6
LV diam.;s (mm)	2.42 ± 0.29	2.37 ± 0.30	2.36 ± 0.29	2.33 ± 0.29	2.64 ± 0.42	2.43 ± 0.37	2.40 ± 0.40	2.20 ± 0.42
LV diam.;d (mm)	3.95 ± 0.31	3.89 ± 0.23	3.92 ± 0.27	3.78 ± 0.17	4.10 ± 0.35	3.91 ± 0.21	4.05 ± 0.38	3.81 ± 0.22
SV (µL)	47.5 ± 8.5	45.7 ± 6.0	47.4 ± 8.5	42.0 ± 6.7	48.5 ± 8.6	44.8 ± 6.1	51.9 ± 13.3	45.2 ± 4.4
FS (%)	39.0 ± 4.4	39.3 ± 5.5	39.8 ± 5.5	38.4 ± 6.9	35.9 ± 6.2	37.9 ± 7.1	40.8 ± 7.7	42.4 ± 8.4
EF (%)	69.7 ± 5.4	69.8 ± 6.8	70.6 ± 6.9	68.6 ± 8.8	65.4 ± 8.2	68.0 ± 9.3	71.4 ± 9.6	73.1 ± 9.5
CO (mL/min)	20.4 ± 4.4	20.2 ± 3.2	22.0 ± 4.4	20.1 ± 3.9	21.5 ± 4.3	21.1 ± 3.7	25.1 ± 6.9	21.9 ± 3.7
IVRT (ms)	18.3 ± 1.8	18.0 ± 2.5	19.2 ± 4.1	18.8 ± 3.1	17.0 ± 2.4	16.7 ± 3.3	17.1 ± 4.9	14.2 ± 2.5
E/A ratio	1.61 ± 0.24	1.69 ± 0.36	1.68 ± 0.55	1.67 ± 0.45	1.79 ± 0.41	1.77 ± 0.37	1.51 ± 0.36	1.94 ± 0.85
E/e' ratio	42.6 ± 11.0	41.1 ± 10.5	41.2 ± 13.4	42.4 ± 13.3	38.1 ± 8.3	35.7 ± 12.0	38.1 ± 8.3	29.0 ± 6.5
E decel. (ms)	30.1 ± 6.5	29.6 ± 5.4	33.7 ± 8.5	29.3 ± 5.5	32.3 ± 8.1	30.3 ± 8.0	28.9 ± 10.0	30.2 ± 5.7
LV MPI	0.69 ± 0.10	0.74 ± 0.14	0.77 ± 0.24	0.70 ± 0.11	0.71 ± 0.20	0.68 ± 0.13	0.72 ± 0.11	0.62 ± 0.10
LA area (mm^2)	6.5 ± 1.1	6.6 ± 0.9	6.5 ± 0.9	7.1 ± 1.0	7.2 ± 1.5	6.8 ± 1.0	7.5 ± 1.5	7.4 ± 1.0

Table 1: Evolution of cardiac function. Cardiac parameters measured by echocardiography in $Ezh2^{fl/fl}$;αMHC-MerCreMer mice before (t_0) and at 1-2 months, 3-4 months and 6-7 months after treatment with 3 × 30 mg/kg of tamoxifen (TAM) or vehicle solution (Con). BW: body weight; LV: left ventricle; vol.: volume, diam.: diameter; SV: stroke volume; FS: fractional shortening; EF: ejection fraction; CO: cardiac output; IVRT: isovolumic relaxation time; decel.: deceleration; MPI: myocardial performance index; LA: left atrial.

Histological analysis focused primarily on the detection of cellular senescence in the myocardium, following recommendation published in recent guidelines (Cell 2019, 179:813). Identification mainly focused on the histological staining for senescence associated β-galactosidase activity (SA-β-Gal) and immunostaining for $p16^{INK4a}$ and p21. Additionally, the staining for γH2AX nuclear foci, as a marker of secondary senescence (not directly induced by the activation of $p16^{INK4a}$ pathway following Ezh2 deletion), was assessed.

Staining of SA-β-Gal was validated on cryosection of liver tissue and white adipose whole tissue, where differential staining intensity were identified in aged mice (~2 years-old) vs. young mice (4 months-old). No SA-β-Gal staining was identified on heart tissue section from either positive and negative control animals, nor from $Ezh2^{fl/fl}$:αMHC-MerCreMer vehicle or tamoxifen treated at any time point. Immunofluorescent staining enabled the identification of $p16^{INK4a}$-positive cardiomyocytes on tissues section obtained to serve as a positive control (Fig. 2). However, no $p16^{INK4a}$-positive cardiomyocytes could be identified in either aged mice or on tissues from $Ezh2^{fl/fl}$:αMHC-MerCreMer mice. Consistently, no other marker of cellular senescence was detected either.

Those observations are consistent with the absence of development of cellular senescence following Ezh2 deletion contrary to the hypothesis this work is based on. Several explanations for this observation were proposed, including the possibility of a compensatory mechanism and an efficient elimination of senescent cardiomyocytes from the heart tissue by the immune system. However, given the difficulty for thorough testing of those hypothesis by design, no satisfactory answer was found.

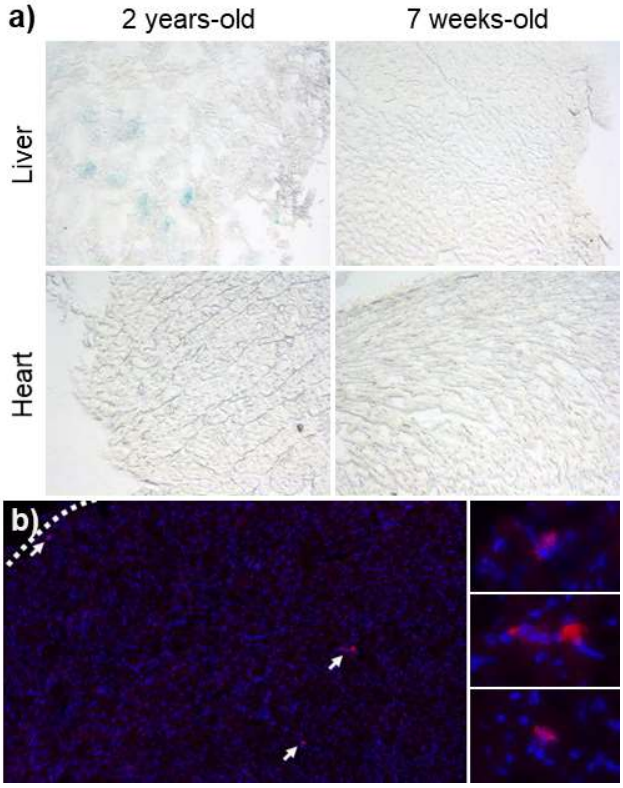

Fig. 2: Validation of cellular senescence markers. a) Detection of global burden of cellular senescence using SA-β-Gal staining on liver and heart tissue section from 2 years-old and 7 weeks-old mice. b) Detection of $p16^{INK4a}$ positive cells in frozen mouse heart tissue from senescence positive control animals.

Succinct methods:

Animals. Homozygote double transgenic $Ezh2^{fl/fl}$:αMHC-MerCreMer and αMHC-MerCreMer animals for Ezh2 conditional deletion and as control of Cre-recombinase toxicity. Animals were injected i.p. with tamoxifen in corn oil solution or vehicle solution (as control) for 3 or 5 consecutive days at doses ranging from 10 to 50 mg/kg.

Genomic recombination. Genomic DNA was extracted form heart tissue (DNeasy Blood & Tissue Kit, Qiagen) and used as DNA template for standard PCR (40 cycles, annealing temperature 60 °C) with 2 sets of primers to detect wild-type and Cre-excision allele; PCR products were run on an 1% agarose gel containing Sybr Safe for qualitative assessment of genomic recombination. Using the same DNA template and primer set, quantitative PCR was performed for quantitative assessment of genomic recombination, following methods adapted from Cell Reports, 2016, 14: 2925.

Echocardiography. Animals underwent echocardiography (Vevo2100, VisualSonics) prior and up to 7 months after tamoxifen (or control) treatment. Mice under 2% isoflurane anesthesia were imaged in short axis, parasternal long axis and by power and tissue doppler to assess various cardiac function parameters including left ventricular ejection fraction and mitral flow and mitral valve

motion during early diastole and atrial contraction. Data are presented as mean ± SD; the differences between the means was assessed by 2-way ANOVA with correction for multiple comparison by controlling the false discovery rate.

SA-β-Gal staining and immunofluorescence. All cardiac and non-cardiac control tissues were harvested after animal euthanasia and deep-frozen in liquid nitrogen of on dry ice prior to tissue embedding in OCT and sectioning. Senescence-associated β-Galactosidase staining was performed using a dedicated kit, following manufacturers' instructions (Senescence Detection Kit, Abcam) For immunofluorescence, the sections were incubated with primary antibodies overnight at 4°C, followed by the appropriate Alexa Fluor 594-conjugated secondary antibodies, counterstained with DAPI and mounted. Tissue section were visualized and digitalized on an epifluorescence microscope (DMi8, Leica) and analyzed using ImageJ.

Aim 2: Development and validation of novel tracer for non-invasive imaging of cellular senescence.

The specific objectives of the tasks to be achieved within the first year of the project in Aim 2 were to prepare the antigen specific to senescent cells: MDA-vimentin and use this antigen to generate single domain antibodies.

The original approach, as presented in the statement of work, was to order recombinant vimentin protein and chemically modify the protein to obtain the targeted antigen: MDA adduct on the cysteine 328, which was observed *in vivo* and reported as specific of cellular senescence.

To that end, an initial approach was to set in place an analytic method capable of identifying the occurrence of the specific modification and absence of undesired modifications, which could create undesired, potentially immunogenic antigen. A method based on LC-MS analysis of peptides obtained from the cleavage of the protein by trypsin, in comparison with *in silico* predicted peptides, was set in place. The implementation of this analytical method, described by Frescas et al. (Proc Natl Acad Sci U S A. 2017, 114(9):E1668), required multiple iteration of testing to find proper conditions for this experimental setting.

In parallel, a series of experiments on amino acid was carried out with the objective of finding optimal condition for specific modification of cysteine residues with MDA. Preparation of MDA was carried out following described methods (J. Am. Chem. Soc. 1981, 103:3030; J. Sci. Food Agric 2009, 89:1416) by acid hydrolysis of 1,1,3,3-tetramethoxypropane. The formation of MDA was confirmed by liquid chromatography–mass spectrometry (LC-MS) analysis (Fig. 2a). The reaction between MDA, either crystallized or freshly prepared, and amino acids (N-acetylated methyl esters of cysteine and lysine) was tested using various concentration ratios, buffer conditions, pH, temperature and oxidative conditions (sodium azide). The progress of the reaction was monitored by LC-MS to identify modified amino acids (Fig. 2b, c). While some conditions showed complete reaction of N-acetyl-L-lysine methyl ester, no condition was found where the modification of N-acetyl-L-cysteine methyl ester was observed. After a failure to obtain the expected modification, it was hypothesized that the reaction on the cysteine would be more favorable in conditions closer related to the *in vivo* situation, with the cysteine engaged in peptide bonds with adjacent residues. A peptide corresponding to the residues 316 to 333 of vimentin

protein was synthetized and similar experiments of reaction with MDA were performed. However, no condition was found where the modification of the cysteine by MDA was observed.

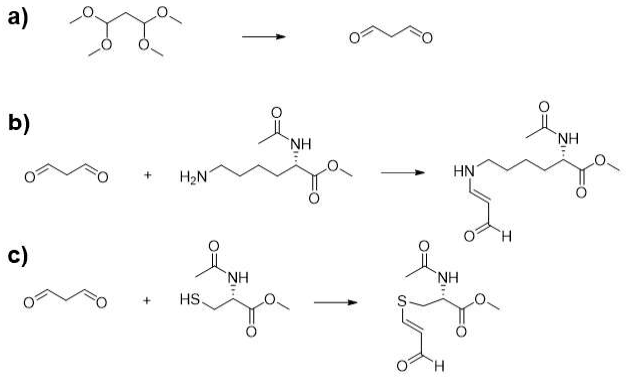

Fig. 2: Model reactions to test MDA modification on amino acids.
a) Preparation of MDA by acid hydrolysis of 1,1,3,3-tetramethoxypropane; **b)** model reaction of MDA with N-acetyl-L-lysine methyl ester, expected to produce MDA modified lysine (m/z: 256.14); **c)** model reaction of MDA with N-acetyl-L-cysteine methyl ester, expected to produce MDA modified cysteine (m/z: 231.06).

The inability to modify cysteine thiol group with the oxidation adduct MDA lead to renounce to the generation of MDA-vimentin as a target. Instead, effort was put to identify an alternative target specific of cellular senescence in the heart. Of note, this change in the molecular target, for imaging the same biological process was not considered as a significant change in the project or its direction, since the all anticipated studies are expected to be perform without alteration to the initial plan with an alternative target.

A first step was an extensive review of the literature to identify potential targets. A total of 24 potential targets: protein expressed at the cell membrane, upregulated in senescent cells, were identified. However, those results were obtained primary in established models of cellular senescence, mainly in fibroblast cell lines. A set of experiments was designed to test the validity of those results in cardiomyocytes, to ensure the relevance for this project. First, cardiomyocyte cell line HL-1 were obtained and amplified. HL-1 cells were treated with doxorubicin to induce cellular senescence. After 1 weeks, cells were harvested, and gene expression analysis was performed. Successful induction of p21-mediated cellular senescence was observed along with the upregulation of several alternative targets identified in the literature review. The highest upregulation in gene expression was observed for *Notch3* (Fig.3, log2 fold change: 4.35).

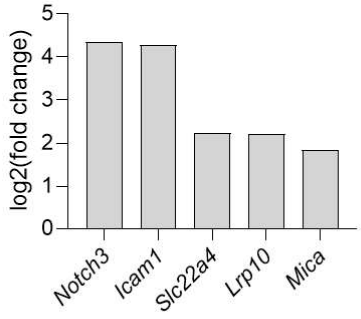

Fig. 3: Gene expression analysis of targets overexpressed in senescent murine cardiomyocytes.
Top 5 overexpressed gene, among the tested 24 target genes, in murine HL-1 cardiomyocytes where cellular senescence was induced by doxorubicin compared to control HL-1. Gene expression was normalized to *Gapdh*.

The validation of the relevance of this target in vivo was pending development of the animal mode of inducible cellular senescence in cardiomyocytes. However, absence of detectable cellular senescence in the proposed animal model (Aim 1) prevented this validation for this target, or other alternative targets. Additionally, immunostaining of cardiac tissue revealed a limited applicability of *Notch3* for the imaging of cellular senescence as a strong expression of this marker in vascular tissue would be potentially an important limiting factor.

Succinct methods:

Chemistry. MDA by acid hydrolysis (1:5 HCl mM) of 1,1,3,3-tetramethoxypropane 8.4 mM (40 °C, 30 min) neutralized to pH 7.5 with NaOH. Amino acid reaction were carried out for different ratio of MDA (up to 0.2 M) to N-acetylated lysine or cysteine methyl esters (or vimentin peptide) in 50 mM PBS or 100 mM acetate buffer, with pH range from 2 to 12, at temperature range from room temperature to 80 °C, in presence or absence of sodium azide 2.5 mM, with reaction time range from 1 h to 18 h (overnight). LC-MS analysis was perform using Agilent LC-MS 6120B Quadrupole, 6490 Triple Quad, 6550A iFunnel Q-TOF.

Cell culture. Murine cardiomyocytes HL-1 cells were cultured on gelatin/fibronectin coated cell culture dishes in Claycomb culture media supplemented with HL-1 qualified FBS, norepinephrine, L-glutamine and penicillin/streptomycin. Cellular senescence was induced by 24 h exposure to doxorubicin 10 μM; senescent cells were harvested at 1 week after the exposure.

Gene expression analysis. Senescent and control cells were lysed and total RNA was extracted and reverse-transcribed to cDNA (Qiagen kits). Quantitative PCR analysis was performed using Sybr Green master mix and appropriate primer sets (7500 Real-Time PCR System, Applied Biosystems). Amplification results were normalized to *Gapdh* ($2^{\Delta CT}$) and analyzed as fold change (senescent to control cells).

What opportunities for training and professional development has the project provided?

Nothing to Report

How were the results disseminated to communities of interest?

Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to Report

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Identification of makers specific of cellular senescence and the validation of their relevance in various tissues is of interest for various application; this is particularly true for makers expressed at the cell membrane and thus potentially of use for targeting senescent cells (Cell 2019, 179:813). Overexpression of Notch3 was previously reported in human fibroblast and cancer cell lines (Cancer Res. 2013, 73:3451; Cell Death Dis. 2014, 5:e1528). Here Notch3 was validated as a marker of cellular senescence induced by doxorubicin in murine cardiomyocytes. However, high expression of Notch3 by vascular endothelial cells restricts its utility as an *in vivo* marker of cellular senescence.

The inability to detect cellular senescence following the deletion of Ezh2 was an unexpected finding in this project. Indeed, it was reported that the disruption of polycomb complex components, such as Ezh2 are sufficient for the activation of $p16^{INK4a}$ and the induction of senescence (J Clin Invest. 2018 1284:1238). While the mechanism behind this observation was not elucidated, this observation is of importance for the study of cellular senescence in cardiac tissue.

What was the impact on other disciplines?

Malondialdehyde (MDA) oxidative adduct on the side chain of cysteine has been reported to occur *in vivo* (Proc Natl Acad Sci U S A. 2017, 114:E1668). However, only sparse literature on MDA chemistry with thiol groups have been reported so far (Free Radic Biol Med. 1991, 11:81). Here a systematic study aiming at the chemically modification of cysteine with MDA was performed and no conditions were found where the desired chemical modification was observed. This negative data is relevant for any further researcher planning to modify cysteine with MDA *in vitro*.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

As described previously, the generated animal model did not present the anticipated phenotype. Henceforth, the pursuit of the studies as described in Aim 1 of the statement of work was not possible, although the cross-sectional evaluation was expended to try to identify conditions where the cardiac specific cellular senescence occurs and include relevant controls.

In Aim 2, different conditions were tested to modify the side chain of cysteine with the oxidation adduct MDA but this approach remained unsuccessful. Therefore, MDA-vimentin, issue from the modification of the recombinant protein (original approach), or MDA-vimentin peptide (considered alternative) could not be generated and used as antigen to generated single domain antibodies. It was decided to pursue with an alternative target. With the failure to produce MDA-vimentin as initially planned, the alternative strategy was refocused to identify relevant targets of cardiac cellular senescence. The evaluation of culture cardiomyocyte identified some alternative targets, which were expected to be validated in vivo, in the generated animal model. With the absence of identifiable cellular senescence in the generated model, those targets could not be validated in vivo. Additionally, the lead candidate identified using the cell assay, Notch3, was shown to have some major limitation for being used as an imaging agent.

Actual or anticipated problems or delays and actions or plans to resolve them

As mentioned above, the main issue at the very core of this project was the absence of cellular detectable senescence in the animals generated to specifically induce cellular senescence in a time controlled and tissue specific manner. Another major problem was the inability to generate MDA-vimentin (or MDA-vimentin peptide) as initially planned, and the inability to validate alternative markers without the animal model.

Changes that had a significant impact on expenditure

Additional experimental group were used over a longer time to try to identify conditions where cellular senescence would be observed; overall, this, and the incorporation of additional control groups increased the duration and number of animals used in the cross-sectional study compared to what was anticipated.

The inability to generate the initially planed marker of senescent cell as intended, lead to an expended systematic study aiming at performing the desired the chemically modification, then, with its failure, to strategies to identify alternative markers, including cell assay using cultured cardiomyocyte, and pilot validation studies (hampered by the lack of in vivo model).

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

  Journal publications.

  Nothing to Report.

  Books or other non-periodical, one-time publications.

  Nothing to Report.

  Other publications, conference papers and presentations.

  Nothing to Report.

- **Website(s) or other Internet site(s)**

  Nothing to Report.

- **Technologies or techniques**

  Nothing to Report.

- **Inventions, patent applications, and/or licenses**

  Nothing to Report.

- **Other Products**

  Generation of $Ezh2^{fl/fl}$;αMHC-MerCreMer mice

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Jakub Toczek
Project Role:	PI
Researcher Identifier (ORCID ID):	0000-0003-0639-8075
Nearest person month worked:	17
Contribution to Project:	Dr. Toczek designed the experiments, provided supervision to the postgraduate associate recruited on the project, participated in animals breeding, experimentation and data interpretation
Funding Support:	W81XWH1910107, 19POST34450142

Name:	Nowshin Sanzida
Project Role:	Postgraduate Associate
Researcher Identifier (ORCID ID):	0000-0002-2432-5489
Nearest person month worked:	13
Contribution to Project:	Ms. Sanzida, participated in animals breeding, experimentation and data interpretation

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

The laboratory INSERM UMR_S 1039 is an academic institution, part of the French national institute for biomedical research (INSERM). This group possess considerable expertise in the development of antibody-based radiotracers.

Organization Name: INSERM UMR_S 1039

Location of Organization: France

Partner's contribution to the project: Consulting on study and experiments design.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Not applicable

QUAD CHARTS:

Not applicable

9. APPENDICES:

None.