Detection of Senescence Markers During Mammalian Embryonic Development

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Summary

Senescence-associated β-galactosidase (SAβ-gal) is a convenient histological technique used to identify senescent cells. Its ease of use is helpful to initially screen and detect senescent cells in heterogeneous cell populations both in vitro and in vivo. However, SAβ-gal staining is not an unequivocal marker of the senescent state, and diagnosis of such usually requires additional markers demonstrating an absence of proliferation and expression of cell-cycle inhibitors. Nonetheless, SAβ-gal remains one of the most widely used biomarkers of senescent cells. Recently, by measuring SAβ-gal activity, the expression of the cyclin-dependent kinase inhibitor p21 (waf1/cip1) and demonstrating a lack of proliferation, we identified senescent cells in the developing embryo. This chapter describes the methods for identifying cellular senescence in the embryo, detailing protocols for detection of SAβ-gal activity in both sections and at the whole mount level, and immunohistochemistry protocols for the detection of additional biomarkers of senescence.

Key Words

Cellular senescence, SAβ-gal, Whole-mount staining, Embryo, Biomarker, Development

1. Introduction

Cellular senescence is an irreversible form of cell cycle arrest that limits the proliferative potential of cells. Initially, senescence was identified in primary human fibroblasts that had undergone replicative exhaustion ***(1)***. Subsequent studies demonstrated that senescence could be induced by a wide variety of stimuli including oncogenic signalling, DNA-damage, oxidative stress and chemotherapeutic drugs ***(2-4)***. In addition to the permanent arrest of cell division, senescent cells exhibit activation of tumour suppressor networks and an altered pattern of gene expression

(5-9). Historically however, while cellular senescence has been best characterized as a tumour suppressive mechanism ***(10-13)*** and a driver of cellular and tissue aging ***(14, 15)***, recent discoveries have extended its known roles, to include beneficial effects during embryonic development ***(16, 17)*** and tissue repair ***(18-20)***.

Twenty years ago, it was discovered that senescent cells express a β-galactosidase activity that is detectable at a sub-optimal pH of 6.0 ***(21)***. This finding introduced a quick and easy way of screening for senescent cells in vitro and in vivo, with the enzymatic activity being distinct from the acidic β-galactosidase activity that is present in all cells at pH4.0. The appropriately named 'Senescence-Associated β-galactosidase' (SAβ-Gal) was detected using a cytochemical assay based on the production of an insoluble blue-dye precipitate that results from the cleavage of X-gal by endogenous β-galactosidase, and which appears to be independent of DNA synthesis ***(21)***. Later, it was elucidated that SA β-Gal activity is due to an increase in the abundance of lysosomal enzymes, possibly linked to an increase in lysosomal biogenesis ***(22)***. Subsequently, it was shown that the increased activity of lysosomal β-D-galactosidase in senescent cells was a direct consequence of increased levels of *GLB1* mRNA and protein ***(23, 24)***. Since then, the presence of SA β-Gal activity has been used extensively to identify senescent cells in a variety of conditions, including in cells in culture reaching the end of their lifespan or undergoing premature senescence, or in vivo in response to a variety of senescence-inducing stimuli ***(11-13, 18, 20, 25, 26)***. However, a note of caution has to be included, as SAβ-gal alone is insufficient as a unique identifier of senescence, as there are incidences where non-senescent cells stain positive or where SAβ-gal activity is not present in senescent cells ***(21, 27, 28)***. As such, the identification of the senescent state requires the use of

additional markers to confirm the status, including an absence of markers of cell proliferation such as BrdU or Ki67, and the expression of hallmark regulatory cell-cycle inhibitors such as p16, p53 or p21.

Here we describe general methods to detect SAβ-Gal and the senescence marker p21 during embryonic development that can be utilised on both chick and mouse embryos with consistent results.

2. Materials

Prepare all solutions using ultrapure water and store all reagents at room temperature unless otherwise stated.

2.1 Dissection of embryos

1. Ethanol: 70% solution in water.
2. Forceps: One pair of blunt and one pair of fine dissecting forceps.
3. Surgical scissors.
4. Petri dishes: 100mm and 35mm.
5. Ice cold Phosphate buffered solution (PBS 1X): 137 mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH7.4.
6. Ice and ice bucket.
7. Stereomicroscope for dissecting embryos.

2.2 Fixation and whole-mount SAβ-Gal staining of embryos

1. Fixative solution: 0.5% glutaraldehyde diluted in PBS, pH7.4 (*see* **Note 1)**.
2. 15mL Falcon tubes.

3. Wash solution: PBS supplemented with 1mM $MgCl_2$, pH5.5 (*see* **Note 2**).
4. 20X Stock KC solution: Dissolve 0.82g of potassium ferricyanide, $K_3FE(CN)_6$, and 1.05g of potassium hexacyanoferrate II trihydrate, $K_4FE(CN)_63H_2O$, in 25mL PBS (*see* **Note 3**).
5. 40X X-Gal stock solution: Dissolve 40 mg of 5-Bromo-4-chloro-3-indolyl β-D-galactopyranosidase (X-gal) in 1mL N,N-dimethylformamide (DMF) (*see* **Note 4**).
6. Incubator or oven at 37 degrees Celsius.
7. 4% formaldehyde made up in PBS or neutral buffered formalin (*see* **Note 5)**.

2.3 Fixation and SAβ-Gal staining of frozen sections

1. Phosphate buffered solution (PBS 1X): 137 mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH7.4.
2. Fixative solution: 0.5% glutaraldehyde diluted in PBS, pH7.4 *(see* **Note 1**).
3. 30% Sucrose: Dissolve 15g of sucrose in 40mL PBS with gentle agitation. Once the sucrose has dissolved, top up the solution to 50mL with additional PBS.
4. Tissue-Tek O.C.T compound.
5. Tissue-Tek Cryomold at desired size.
6. Dry Ice & ice bucket**.**
7. Fisher SuperFrost Plus glass slides.
8. 0.5% gelatine.
9. 0.5g Chromium Potassium Sulphate (*see* **Note 6**).
10. Whatman Filter paper.
11. PAP pen.

12. Wash solution: PBS supplemented with 1mM $MgCl_2$, pH5.5 (*see* **Note 2**).
13. 0.2M $K_3Fe(CN)_6$: Dissolve 0.66g of potassium ferricyanide $K_3Fe(CN)_6$ in 10mL of H_2O (*see* **Note 3**).
14. 0.2M $K_4Fe(CN)_63H_2O$: Dissolve 0.85g of potassium hexacyanoferrate II trihydrate, $K_4Fe(CN)_63H_2O$ in 10mL of H_2O (*see* **Note 3**).
15. 40X X-Gal stock solution: Dissolve 40 mg of 5-Bromo-4-chloro-3-indolyl β-D-galactopyranosidase (X-gal) in 1mL N,N-dimethylformamide (*see* **Note 4**).
16. Incubator or oven at 37 degrees Celsius.
17. Eosin.
18. Glass coverslips.
19. Permanent mounting media such as PermaFluor.

2.4 Immunohistochemistry

1. Incubator or oven at 65 degrees Celsius.
2. Coplin jars or glass containers.
3. Xylene (*see* **Note 7**).
4. Ethanol: 100%, 96%, 70% and 50%
5. Distilled water.
6. Phosphate buffered solution (PBS 1X): 137 mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH7.4.
7. Citrate buffer stock solution (10X): Dissolve 1.9g of citric acid monohydrate in 90mL of water (0.02M) and 12.05g of tri-sodium citrate in 410mL water (0.08M). Add the two solutions together and adjust the pH to 6.0 with 10M NaOH and 6M HCl *(see* **Note 8**).
8. Hotplate.

9. 1L glass beaker.
10. Plastic coplin jar.
11. 30% Hydrogen peroxide aqueous (*see* **Note 9**).
12. Triton-X 100.
13. Blocking Solution: 10% goat serum diluted in PBS pH7.4 with 0.1% Triton-X 100.
14. Antibody Dilution Buffer: 1% goat serum diluted in PBS, pH7.4 with 0.1% Triton-X.
15. VectaStain ABC Kit.
16. DAB Substrate Kit (Vector Laboratories).
17. Haemotoxylin counter-stain.

Methods

All procedures should be carried out at room temperature unless otherwise specified.

3.1 Dissection of embryos

1. Euthanatize a timed pregnant mouse at the required stage according to your approved Institutional Animal Care and Use protocol.
2. Placing the pregnant mouse on her back, dab the abdomen with 70% ethanol.
3. Using blunt forceps, lift the skin over the abdomen and make a small lateral incision at the midline with sharp surgical scissors.
4. Enlarge the incision pulling the skin apart to expose the abdomen. Carefully pull out the uterus and remove the uterine horn containing the embryos with the placenta and yolk sac attached, and intact, into a 100mm Petri dish filled with ice-cold PBS, on ice.

5. Separate each embryo by cutting between implantation sites along the uterine horn.
6. Transfer a single embryo to a 35 mm Petri dish filled with ice-cold PBS, and using a stereoscopic microscope and fine forceps, separate the muscular wall of the uterus, Reichert's membrane, the visceral yolk sac and the amniotic sac surrounding the embryo (*see* **Note 10)**.
7. Once the embryo is free from all membranes, transfer back to a new 10cm Petri dish containing fresh ice cold PBS on ice and continue this process until the desired number of embryos are dissected. Leave embryos in PBS on ice for at least 20 minutes to ensure that embryos are adequately euthanatized.

3.2 Fixation and whole-mount SAβ-Gal staining of embryos

1. Fix newly dissected embryos in 0.5% glutaraldehyde diluted in PBS (pH7.4), with approximately 5 – 10mL per embryo, overnight at 4 degrees Celsius with gentle agitation (*see* **Note 11)**.
2. The following day, wash embryos twice in PBS, pH5.5 supplemented with 1mM $MgCl_2$ for 15 minutes each wash with gentle agitation.
3. Prepare the X-gal staining solution by adding 1X X-gal (0.25mL), 1X KC (0.5mL) to 9.25mL of PBS supplemented with 1mM $MgCl_2$, pH5.5 (*see* **Note 2)**.
4. Incubate embryos in the X-gal staining solution at 37 degrees Celsius (without CO_2) for 3 – 6 hours, depending on the stage of the embryo. Keep solution protected from light by wrapping tube with aluminium foil (*see* **Note 12**).
5. Following staining, wash embryos 3 X with water and image using a stereomicroscope equipped with a camera (*see* **Note 13) (Fig.1)**.

6. For extended storage, embryos can be placed in 4% paraformaldehyde or neutral buffered formalin at 4 degrees in the dark, without losing too much apparent staining (*see* **Note 14**).

3.3 Fixation and SA β-Gal staining of frozen embryonic sections

1. Obtain embryonic tissue of interest and wash with PBS to remove any residual blood or unwanted membranes.
2. Fix tissue in 0.5% glutaraldehyde diluted in PBS (pH7.4), overnight at 4 degrees Celsius with gentle agitation (*see* **Note 11)**.
3. After fixation, wash tissue twice in PBS for 15 minutes each wash, with gentle agitation.
4. Place tissue in 30% sucrose diluted in PBS (pH7.4), overnight at 4 degrees Celsius with gentle agitation.
5. Place embryonic tissue in a Tissue-Tek Cryomold and apply O.C.T. compound in sufficient quantity to completely cover the tissue. Subsequently place the cryomold on top of dry ice allowing the O.C.T. to freeze (*see* **Note 15**).
6. Prepare 0.5% gelatine by adding 5 grams of gelatine to 200mL of warm water and stir until dissolved. Bring the volume of the solution up to 1L using distilled water and add 0.5g of chromium potassium sulphate to the solution. Once dissolved, filter the mixture using Whatman paper and dip glass slides into the solution for 1 minute. Drain slides on a tray lined with paper towels and place in an oven at 37 degrees Celsius until dry.
7. Cut 10μm sections and place onto slides (Fisher Superfrost Plus) that have been treated with 0.5% gelatine to make them adhesive. Samples can be stored

at -80 degrees Celsius for a period up to one week however the enzyme is not particularly stable and samples should be processed quickly.

8. Remove desired slides from -80 degree Celsius freezer and place in an oven or incubator at 37 degrees Celsius for 20 minutes to remove any residual water from the sections**.**
9. Wash slides for 5 minutes in PBS pH7.4 to remove O.C.T. compound.
10. Using a PAP pen, outline the tissue sections on the glass slide**.**
11. Wash sections twice in PBS, pH5.5 supplemented with 1mM $MgCl_2$ for 5 minutes each wash.
12. Prepare the X-gal staining solution by adding 1X X-gal (0.25mL), 0.2M $K_3Fe(CN)_6$ (0.25mL) and 0.2M $K_4FE(CN)_6 3H_2O$ (0.25mL) to 9.25mL of PBS supplemented with 1mM $MgCl_2$, pH5.5 (*see* **Note 2**).
13. Incubate sections in the X-gal staining solution at 37 degrees Celsius (without CO_2) for 4 – 6 hours, in a humid chamber. Keep solution protected from light by wrapping with aluminium foil (*see* **Note 16**).
14. Following staining, wash sections 3X in PBS for 5 minutes each wash.
15. Counterstain with Eosin.
16. Add several drops of mounting media along the slide and place a glass coverslip on the slide. Staining of sections can be observed under a light microscope.

3.4 Immunohistochemistry of paraffin embedded sections

1. Isolate tissue of interest, fix and embed in paraffin according to standard histology procedures. For embryonic tissues, optimization of fixation time may be required. Cut sections as required, and mount on glass slides.

2. Place slides with sections of paraffin embedded tissue in a 65 degree Celsius oven for 20 minutes to aid removal of the paraffin.
3. Wash slides twice in xylene in a glass coplin jar for 10 minutes each wash in a fume hood.
4. Rehydrate sections by washing slides for 5 minutes in the following series of solutions: 100% Ethanol, 100% Ethanol, 96% Ethanol, 70% Ethanol, 50% Ethanol and distilled water.
5. Wash slides twice for 5 minutes in PBS pH7.4.
6. Prepare 1X citrate buffer by adding 100mL of 10X stock solution to 900mL of distilled water. Ensure the pH remains at 6.0. Preheat the 1X citrate buffer to 95 – 100 degrees Celsius in a plastic coplin jar by placing in a water bath and transfer sections into the pre-heated buffer for 20 minutes.
7. Remove the coplin jar from the water bath and retain the sections in the solution for 30 minutes, allowing the solution to cool to room temperature.
8. Wash slides twice for 5 minutes in PBS pH7.4.
9. Quench sections with 0.3% hydrogen peroxide diluted in PBS for 15 minutes.
10. Wash slides twice for 5 minutes in PBS pH7.4.
11. Permeabilise sections in PBS pH7.4 with 0.2% Triton-X for 10 minutes.
12. Wash slides twice for 5 minutes in PBS pH7.4.
13. Using a PAP pen, outline the tissue sections on the glass slide and add blocking solution (10% goat serum in PBS, pH7.4 with 0.1% Triton-X) to the sections for 1 hour (*see* **Note 17**).
14. Incubate slides with primary antibody diluted in 1% goat serum in PBS, pH7.4 with 0.1% Triton-X) overnight at 4 degrees Celsius in a humid chamber. Antibody concentration may require optimization. For p21 staining in

embryos, anti-p21 antibody, (SXM30, BD Pharmingen) was used at a concentration of 1:200.

15. Wash slides twice for 5 minutes in PBS pH7.4.
16. Add biotinylated secondary antibody diluted in 1% goat serum in PBS, pH7.4 for 1 hour.
17. Wash slides twice for 5 minutes in PBS pH7.4.
18. Incubate for 30 minutes with VectaStain ABC Reagent.
19. Wash slides twice for 5 minutes in PBS pH7.4.
20. Develop reaction with DAB Kit (*see* **Note 18**).
21. Wash slides twice for 5 minutes in PBS pH7.4.
22. Counter-stain samples with haematoxylin and wash slides for 5 minutes in water.
23. Dehydrate sections by washing slides for 30 seconds in the following series of solutions: 96% Ethanol, 96% Ethanol, 96% Ethanol, 100% Ethanol, 100% Ethanol, xylene and xylene.
24. Mount sections in non-water soluble mounting medium.

Notes

1. Glutaraldehyde can be bought commercially as a 50% aqueous solution. It is both toxic and a strong irritant. Aliquots should be made in a fume hood, with the user wearing appropriate personal protective equipment and stored at -20 degrees Celsius to avoid repeat thawing and freezing of the solution.
2. The optimal pH of the PBS supplemented with $MgCl_2$ is a critical step to obtain correct results. For staining mouse tissue or chick embryos, pH 5.5 is optimal: however if staining human samples, pH 6.0 is needed.

3. This solution must be protected from light, by wrapping with aluminium foil, and can be stored up to 1 month at RT. Potassium ferricyanide and potassium hexacyanoferrate II trihydrate both have relatively low toxicity while in powdered form, with the main hazard being mild irritation to the eyes and skin. However, under very strong acidic conditions, a highly toxic cyanide gas can be omitted. Therefore appropriate storage of these chemicals away from acids is highly recommended.
4. We find that it is best to prepare this fresh each time. 5-Bromo-4-chloro-3-indolyl β-D-galactopyranosidase (X-gal) is also relatively expensive therefore depending on the number of embryos being stained it may be prudent to only make the amount of 40X X-gal required. Fresh DMF is also recommended.
5. If making 4% formaldehyde from paraformaldehyde powder, be very careful when manipulating the powder. Paraformaldehyde is considered a carcinogen and the powder is very fine and easily inhaled. For 1L, add 800mL of 1X PBS to a glass beaker on a stir plate in a ventilated hood and heat PBS while stirring to approximately 60 degrees Celsius. Carefully weigh 40g paraformaldehyde powder using a mask and other personal protective equipment and add to the heated PBS solution. Initially the powder will not dissolve. Next, slowly raise the pH by adding 1M NaOH drop wise until the solution clears. Allow the solution to cool and filter using a vacuum filtration unit. Finally, adjust the volume of the solution to 1L with 1X PBS and make aliquots to be stored at -20 degrees Celsius until needed.
6. Chromium Potassium Sulphate is an irritant and may be harmful if absorbed through the skin. This chemical should be handled in a fume hood, with the user wearing appropriate personal protective equipment.

7. Xylene is both flammable in liquid and vapour forms and is extremely toxic. Single exposure can cause respiratory irritation and if swallowed may be fatal. This liquid should be handled with extreme caution, in a fume cabinet and the user must wear appropriate personal protective equipment. Additionally, xylene is a solvent and should only be poured into glass or appropriate plastic compatible containers.
8. Both NaOH and HCl are corrosive reagents and should only be handled wearing appropriate personal protective equipment.
9. Hydrogen peroxide is thermodynamically unstable and will decompose to form water and oxygen therefore do not use the 30% stock solution if older than 3 months.
10. It is imperative that all membranes are removed from the embryo as residual membranes will not allow the staining solution to properly permeate the embryo and inconsistent results may be obtained.
11. It is possible to fix embryos in a combination of 0.2% glutaraldehyde and 2% formaldehyde diluted in PBS that may aid in co-immunostaining on sections or with performing SAβ-gal staining on sections after cutting ***(17)***. We found that fixation in 0.5% glutaraldehyde gives a very robust signal superior to the combined glutaraldehyde/ formaldehyde fixation. However glutaraldehyde is a very strong fixative and makes co-staining with antibodies very difficult. Please note, that fixation times should be optimized for different tissues or different stages of embryonic development, and longer than 16 hours of fixation in 0.5% glutaraldehyde may lead to over-fixation of the tissue and may actually quench the enzymatic activity of the X-gal reaction.

12. It is possible to embed the stained embryos in paraffin and subsequently section the embryos following whole mount SAβ-gal staining **(Fig. 2)**. This may be improved by keeping the embryos in X-gal staining solution longer, or overnight at 37 degrees Celsius, before proceeding to fixation. The embryos will generally have some light blue background staining ("over-staining"). However during the process of paraffin embedding this background staining will be cleared.
13. Embryos should be imaged immersed in PBS and within a maximum of 6 hours following staining and with as minimal exposure to white light as possible. Leaving embryos overnight in PBS will result in an increase in a non-specific light bluish background staining.
14. Following overnight fixation in 4% formaldehyde, embryos can be manually dehydrated by immersing the embryo twice for 15 minutes through a series of 50%, 70%, 96% and 100% ethanol. Embryos are then placed in two washes of xylene for 7.5 minutes each wash and then infiltrated by paraffin as per standard embedding practices. We found that using automated paraffin processing procedures removed the X-gal staining which is readily dissolved to some degree in both ethanol and xylene.
15. Use a hammer to crush dry ice so that it becomes powder like. This will create a flat surface in which to place your Cryomold ensuring that the sample remains positioned well as the O.C.T. freezes.
16. SAβ-gal staining may become visible after a few hours, therefore include a control section and check this section for staining after 3 hours and every hour thereafter for absence of background staining. Incubating the tissues for too long in SAβ-gal may result in non-specific background staining.

17. Senescent cells tend to have high background and therefore make immunostaining difficult. The success of immunostaining with classical markers of senescence is dependent on the quality of the primary and secondary antibody. Alternative optimization may include blocking in 5% BSA with 0.1% Triton-X diluted in PBS.
18. DAB is a hazardous chemical and harmful if swallowed, inhaled or placed in contact with the skin. When finished using the DAB solution, place in 10% bleach to neutralize and dispose of in an appropriate manner.

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References

1. Hayflick, L. and P.S. Moorhead, *The serial cultivation of human diploid cell strains.* Exp Cell Res, 1961. **25**: p. 585-621.
2. Serrano, M., et al., *Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a.* Cell, 1997. **88**(5): p. 593-602.
3. Kuilman, T., et al., *The essence of senescence.* Genes Dev, 2010. **24**(22): p. 2463-79.
4. Schmitt, C.A., et al., *A senescence program controlled by p53 and p16INK4a contributes to the outcome of cancer therapy.* Cell, 2002. **109**(3): p. 335-46.
5. Narita, M., et al., *Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence.* Cell, 2003. **113**(6): p. 703-16.
6. Zhang, R., et al., *Formation of MacroH2A-containing senescence-associated heterochromatin foci and senescence driven by ASF1a and HIRA.* Dev Cell, 2005. **8**(1): p. 19-30.
7. Acosta, J.C., et al., *Chemokine signaling via the CXCR2 receptor reinforces senescence.* Cell, 2008. **133**(6): p. 1006-18.

8. Coppe, J.P., et al., *The senescence-associated secretory phenotype: the dark side of tumor suppression.* Annu Rev Pathol, 2010. **5**: p. 99-118.
9. Kuilman, T., et al., *Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network.* Cell, 2008. **133**(6): p. 1019-31.
10. Braig, M., et al., *Oncogene-induced senescence as an initial barrier in lymphoma development.* Nature, 2005. **436**(7051): p. 660-5.
11. Collado, M., et al., *Tumour biology: senescence in premalignant tumours.* Nature, 2005. **436**(7051): p. 642.
12. Chen, Z., et al., *Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis.* Nature, 2005. **436**(7051): p. 725-30.
13. Michaloglou, C., et al., *BRAFE600-associated senescence-like cell cycle arrest of human naevi.* Nature, 2005. **436**(7051): p. 720-4.
14. Baker, D.J., et al., *Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders.* Nature, 2011. **479**(7372): p. 232-6.
15. Campisi, J., *Aging, cellular senescence, and cancer.* Annu Rev Physiol, 2013. **75**: p. 685-705.
16. Storer, M., et al., *Senescence is a developmental mechanism that contributes to embryonic growth and patterning.* Cell, 2013. **155**(5): p. 1119-30.
17. Munoz-Espin, D., et al., *Programmed cell senescence during mammalian embryonic development.* Cell, 2013. **155**(5): p. 1104-18.
18. Krizhanovsky, V., et al., *Senescence of activated stellate cells limits liver fibrosis.* Cell, 2008. **134**(4): p. 657-67.
19. Demaria, M., et al., *An essential role for senescent cells in optimal wound healing through secretion of PDGF-AA.* Dev Cell, 2014. **31**(6): p. 722-33.
20. Jun, J.I. and L.F. Lau, *The matricellular protein CCN1 induces fibroblast senescence and restricts fibrosis in cutaneous wound healing.* Nat Cell Biol, 2010. **12**(7): p. 676-85.
21. Dimri, G.P., et al., *A biomarker that identifies senescent human cells in culture and in aging skin in vivo.* Proc Natl Acad Sci U S A, 1995. **92**(20): p. 9363-7.
22. Kurz, D.J., et al., *Senescence-associated (beta)-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells.* J Cell Sci, 2000. **113 (Pt 20)**: p. 3613-22.
23. Lee, B.Y., et al., *Senescence-associated beta-galactosidase is lysosomal beta-galactosidase.* Aging Cell, 2006. **5**(2): p. 187-95.
24. Debacq-Chainiaux, F., et al., *Protocols to detect senescence-associated beta-galactosidase (SA-betagal) activity, a biomarker of senescent cells in culture and in vivo.* Nat Protoc, 2009. **4**(12): p. 1798-806.
25. Baker, D.J., et al., *Opposing roles for p16Ink4a and p19Arf in senescence and ageing caused by BubR1 insufficiency.* Nat Cell Biol, 2008. **10**(7): p. 825-36.
26. Keyes, W.M., et al., *p63 deficiency activates a program of cellular senescence and leads to accelerated aging.* Genes Dev, 2005. **19**(17): p. 1986-99.
27. Brady, C.A., et al., *Distinct p53 transcriptional programs dictate acute DNA-damage responses and tumor suppression.* Cell, 2011. **145**(4): p. 571-83.
28. Huang, T. and J.A. Rivera-Perez, *Senescence-associated beta-galactosidase activity marks the visceral endoderm of mouse embryos but is not indicative of senescence.* Genesis, 2014. **52**(4): p. 300-8.

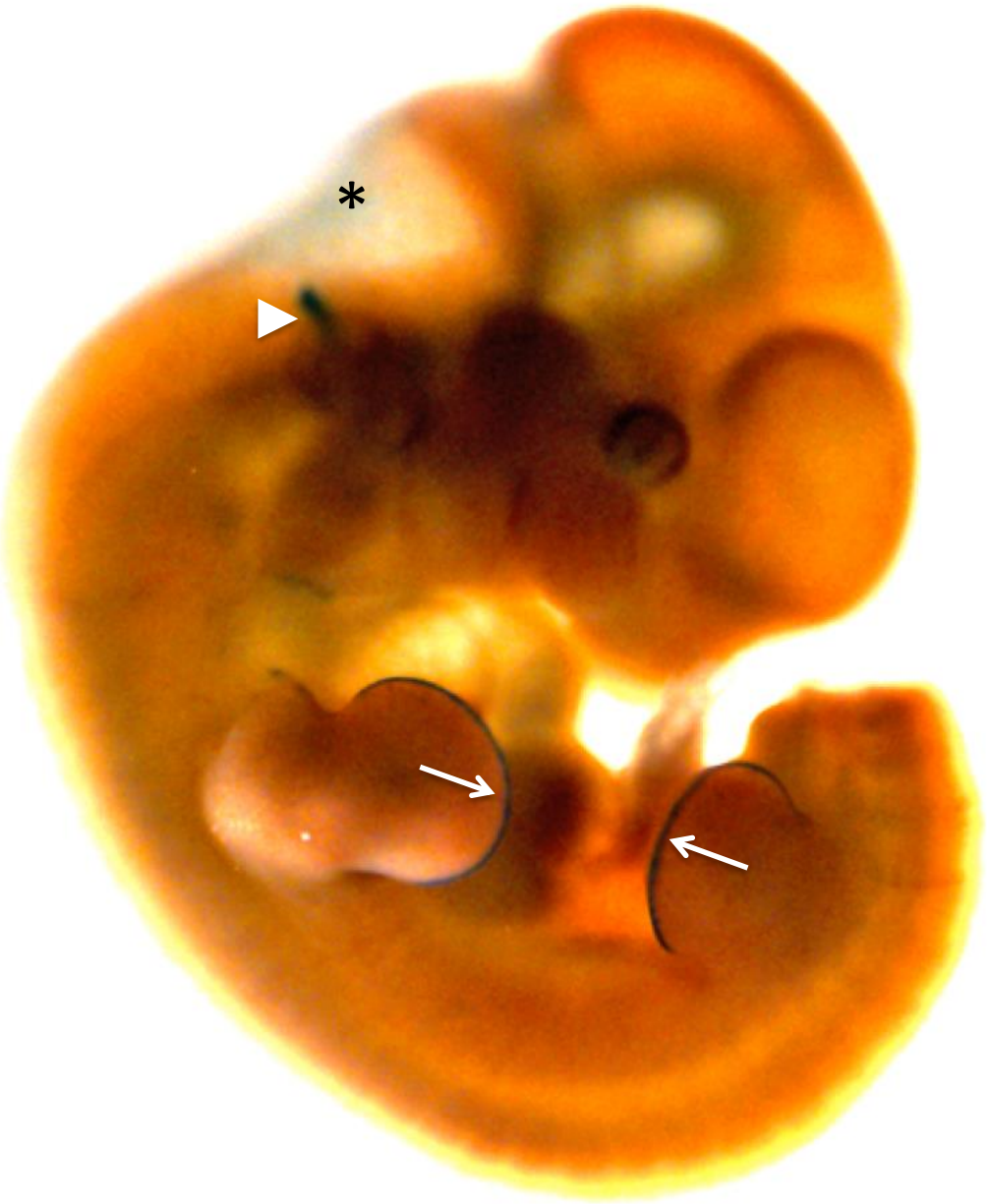

Figure 1. Wholemount SA-gal staining on an embryonic day 11.5 mouse embryo shows senescence staining in the apical ectodermal ridge of the developing limbs (arrows), the otic vesicle (arrowhead) and in the hindbrain (asterisk).

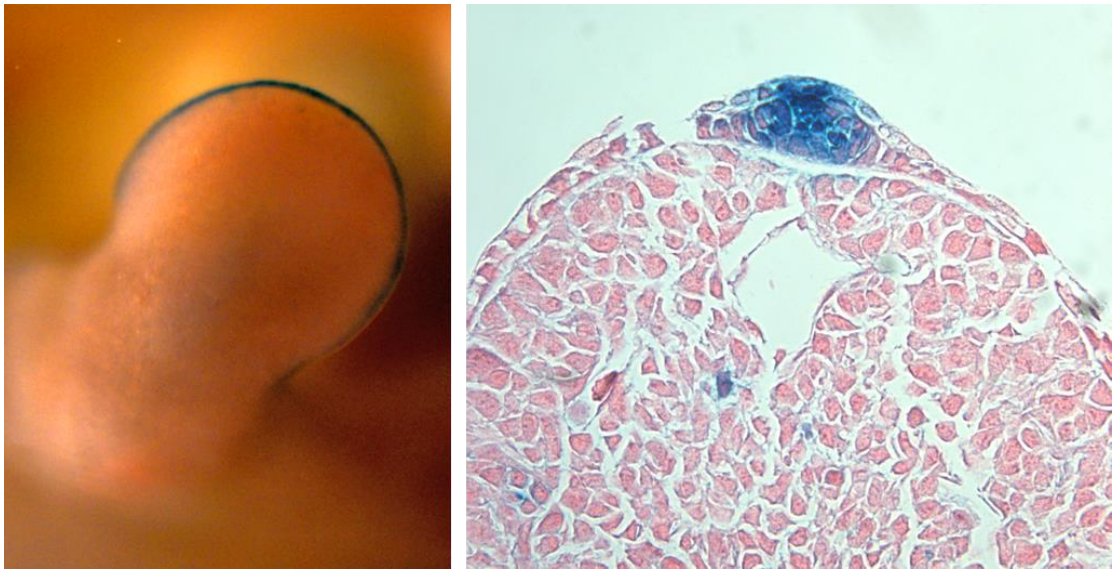

Figure 2. Wholemount SA-gal staining on an embryonic day 11.5 mouse embryo identifies senescence staining in the apical ectodermal ridge of the forelimb (left). Subsequent sectioning of the stained tissue reveals the pattern of staining in the apical ectodermal ridge (right).