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Differential Senescence in Feto-Maternal Tissues During Mouse Pregnancy

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Abstract

Background—Human studies show that fetal membranes have a limited lifespan and undergo telomere-dependent cellular senescence that is augmented by oxidative stress and mediated by p38 mitogen activated protein kinase (MAPK). Further, these studies suggest that fetal membranes are anatomically physiologically positioned to transmit senescence signals that may initiate parturition at term.

Methods—Longitudinal evaluation of feto-maternal tissues from mouse pregnancies was undertaken to determine the molecular progression of senescence during normal pregnancy. On days 10–18 of gestation, C57BL/6 mice were euthanized. Fetal membranes, placenta, and decidua/uterus were collected. Tissues were examined for Telomere length (TL) and the presence of Phosphorylated (P) p38MAPK and p53, p21 and senescence associated β -Galactosidase (SA- β -Gal).

Findings—Telomere length negatively correlated with gestational age in fetal membranes ($\beta = -0.1901 \pm 0.03125$, $p < 0.0001$), placenta (-0.09000 ± 0.03474 , $p = 0.0135$), and decidua/uterus (-0.1317 ± 0.03264 , $p = 0.0003$). Progressive activation p38MAPK was observed in all tissues from days 10 to day 18, with the highest activation observed in fetal membranes. Activation of p53 was progressive in fetal membranes. In contrast, active p53 was constitutive in placenta and decidua/uterus throughout gestation. Detection of p21 indicated that pro-senescent change was higher in all compartments on day 18 as compared to other days. The number of SA- β -Gal positive cells increased in fetal membranes as gestation progressed. However, in placenta and uterus and decidua/uterus SA- β -Gal was seen only in days 15 and 18.

Conclusions—Telomere dependent p38 and p53 mediated senescence progressed in mouse fetal membranes as gestation advanced. Although senescence is evident, telomere dependent events

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Conflict of interest

The authors have no competing interests to declare.

were not dominant in placenta or decidua/uterus. Fetal membrane senescence may significantly contribute to mechanisms of parturition at term.

Keywords

Aging; parturition; mouse; fetal membranes; telomere; p38MAPK; p53

1. Introduction

The length of gestation and parturition in humans and other mammals is determined by gene-by-environment interactions, which ultimately lead to catastrophic failure in functioning of the fetal membranes, leading to uterine contractions, cervical ripening and dilation, and expulsion of the fetus[1]. This is likely a physiologic process designed to promote parturition. Although debate continues, it is thought that the signals leading to parturition may be of both fetal and maternal origin[2]. It is generally thought that a fetal signal occurs when the fetus achieves a certain developmental stage, at which time it no longer requires the protection and support of in utero life, specifically the support provided by placenta and fetal membranes for fetal growth, development and protection[1]. The exact source of the “fetal signal” is unknown, but may include surfactant protein[3] cell free DNA[4], and other hormonal[5], mechanical[6] and immune[7] signals.

We have shown that human fetal membranes (amniochorion) undergo a developmental program that involves the generation of a group of “fetal” signals that initiate or augment mechanisms leading to parturition[8, 9]. This developmental program is senescence[10], a mechanism associated with aging of the intrauterine tissues determining their longevity of in utero life. The signals generated from senescent feto-maternal tissues include a large group of senescence associated factors collectively called as senescence associated secretory phenotype (SASP) [11].

Senescence is the process of cellular aging by which cells change morphologically (size, shape, and organelle structure[12, 13]), in gene (p16Ink4[14], and p21[10]) and protein (beta gal[15]) expression, and in the activation of key signaling constituents, such as p38[16] and p53 [17, 18] that can determine the fate of a tissue. Although it has been postulated that cellular apoptosis transmits an inflammatory signal that promotes parturition[4], senescent cells may be resistant to apoptosis[19], and still transmit both inflammatory and senescence-promoting signals. Thus senescence may comprise a broader array of cell signals relevant to induction of both term and preterm parturition. We have demonstrated senescence associated changes to fetal tissues and SASP signature in human parturition[8]. One of the mediators of senescence associated inflammation is oxidative stress experienced at term due to increased metabolic demand of the growing fetus, depletion of antioxidants or reduced maternal supply of substrates required for redox balance[20]. It is likely that other factors such as increased stretch [21], endocrine changes and fetal stressors can also contribute to this process. Therefore, we posit that oxidative stress at term can perturb homeostatic balances of the intrauterine cavity, promoting senescence associated inflammation that can trigger parturition. Several issues remain in testing this hypothesis and in understanding the potential underlying mechanisms. One is a clear sense of the kinetics of senescence

development and propagation of inflammatory signals and another is the relative impact of senescence in other fetal and maternal tissues. Yet another is the exact delineation of mediators/ modifiers of this process. These are difficult to study in humans due to impracticality of longitudinal sampling and therefore ‘cause/effect’ will remain to be answered. Although in vitro studies have recapitulated many of these findings and addressed many of the mechanistic events associated with senescence associated parturition signals, models are limited due to their inability to accommodate multitudes of in vivo environment. To further address these issues, we have elected to perform a study in normal mouse fetal membranes, placenta, and decidua/uterus. By several parameters we find increased senescence in fetal membranes from mid gestation onward.

2. Materials and methods

2.1. Mice

C57BL/6 mice (aged 3–5 months) were obtained from Jackson Laboratory and housed under specific pathogen free and American Association for Laboratory Animal Care- accredited conditions. Animal use was under protocol approved by the Institutional Animal Care and Use Committee at the University of Vermont. Females and males were timed-mated as previously described [22]. Males and seasoned females were housed together overnight (16 hours) and the males removed the next morning (designated as day 0 of gestation).

2.2. Collection of gestational tissues from different gestational age groups [23, 24]

Pregnant uteri were removed and placed in sterile IMDM (Invitrogen). The uteri were cut longitudinally on the side opposite the implantation sites. Individual fetal-placenta units with associated amniotic sacs and underlying uterus were isolated, and the membranes grasped at the edge of the placenta with a fine forceps and peeled away. Then the placenta was removed from the uterus. If there was a well-defined decidua basalis cap [23], then we attempted to remove it entirely and this was used as the “decidua”. If this was not possible, we used a sample of uterus just below the implantation site after peeling off the placenta. Thus the decidual samples are labeled “decidua/uterus” and, despite careful dissection, these samples have possibly been contaminated with either trophoblast or non-decidual uterine tissue, including myometrium.

2.3. Telomere length measurement

Fetal membranes, placenta and decidua/uterus collected on gestational day 9, 10, 12, 15, 17 and 18 of mouse pregnancies were subjected to a semiquantitative telomere length measurement[25]. DNA isolation was performed using a commercial kit (DNeasy Blood and Tissue Kit, Qiagen, Germantown, MD) following the manufacturer’s recommendations. The quality and concentration of extracted DNA were determined by 260/280 nm absorbance ratio (Gen5, Epoch, Bio Tek, Winooski, VT, USA), and the relative concentration of telomere fragments was analyzed using quantitative real-time PCR (qPCR). References for relative number of telomere fragments were generated by performing serial dilutions from a reference DNA sample to produce concentrations of DNA ranging from 30 to 0.315 ng/μL. triplicate (for standard curves) and duplicate (for samples) PCR reactions using 5 ng DNA for each sample were carried out in a 20μL volume using 2x DNA Master SYBR Green kit

(Applied Biosystems (ABI), Foster City, CA, USA) on an ABI 7500 real-time PCR machine with SDS software, version 1.3.1. Primers for telomere (*tel1b*, 5'-CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT-3'; and *tel2b*, 5'-GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT-3') were added to the final concentrations of 0.2 μ M. The thermal cycling profiles were as follows: 95°C for 10 min, followed by 20 cycles of 95°C for 5s, 56°C for 10s, and 72°C for 60s. No template controls were included in all plate reactions. The relative number of telomere fragments in each specimen was normalized to the reference sample [$2^{-(Ct(\text{sample}) - Ct(\text{control}))} = 2^{-Ct}$].

2.4. Western blot analysis

Fetal membranes, placenta and decidua/uterus from various gestational days were homogenized in RIPA buffer with protease inhibitors using a bullet blender (Next Advance, Averill Park, NY). Protein quantification was done using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL). Samples containing 45 μ g of protein were separated by SDS-gel electrophoresis (Bio-Rad, Hercules, CA) according to manufacturer's suggestions and proteins were transferred to a PVDF membrane using the iBlot dry blotting system (Life Technologies, Grand Island, NY). The membranes were blocked for two hours in 5% milk in TBS-Tween-20. The blots were then incubated with primary antibody to total p38 (Cell Signaling #9212, Danvers, MA), P-p38 (Cell Signaling #9211, Danvers, MA) or P-p53 (ab1431, Abcam, Cambridge, MA), and total p53 (ab31333, Abcam) overnight at dilutions of 1:1000, 1:400, 1:200 and 1:400, respectively. Blots were then washed and incubated with secondary antibody for 1 hour and exposed using Luminata Forte Western HRP substrate (Cat# WBLUF0100) from Millipore (Billerica, MA). In order to avoid inter-assay variability between blots, samples from the same experiments were run on the same gel for a given marker. The blots were all reprobated with antibodies to β -actin (Sigma, St. Louis, MO).

2.5. Senescence Detection by Senescence associated β -galactosidase (SA β -Gal) staining

Fetal membranes, placenta and decidua/uterus from various gestational days were flash frozen and stored at -80°C until they were embedded in OZT (Sakura Finetek, Torrance, CA) and frozen until tissues were sectioned for staining. Cryostat sectioning was performed on tissues (6 μ mbedded in OZT (Sakura Finetek, Torrance, CA) and frozen until tissues were sectioned for staininSA β -Gal staining in a distinct pH (6.0) using a histochemical staining kit in frozen tissue sections (BioVision Senescence Detection Kit, Milpitas, CA). Frozen tissue sections were fixed with 0.5 ml of fixative solution for 15 minutes, at room temperature. Staining was performed on frozen section as per manufacturer's protocol and counterstained lightly with eosin to better visualize cellular morphologies. Number of senescent cells per high power field was examined and a semi quantitative assessment of the number of cells staining for SA β -Gal (blue staining) was performed. Ten high-power fields were examined for both amnion and chorion layers separately and number of positive (blue staining) and negative cells (only eosin stained) were counted.

2.6 Data analysis

Data for telomere length was plotted as a function of gestational age and observed for trends. Data from Western blots to detect activated p38 and p53 was converted to densitometry units. Raw and $\log_{10}$ -transformed densitometry data was also plotted as a function of

gestational age and observed for trends. The relationship between telomere length or log-transformed densitometry data and gestational age was modeled using linear regression with the program available in GraphPad Prism (La Jolla, CA 92037 USA). Beta coefficients for the slope of the lines, R^2 and testing for significance based on these slopes being different from zero are reported. Modeling of published decidual gene expression array data[26] mentioned in the discussion was also accomplished using linear regression.

3. Results

3.1. Telomere length decrease correlates with gestational age in mice

Telomere length was measured in fetal membranes, placenta and decidua/uterus using PCR based approaches that are validated and well reported and validated in our laboratory for gestational tissues. Telomere length showed a decline in both fetal and maternal compartments. Data demonstrated that decrease telomere length in fetal membranes was highly correlated with gestational age ($R^2=0.51$) (Figure 1A) compared to placenta ($R^2=0.15$) (Figure 1B) and decidua/uterus ($R^2=0.34$) (Figure 1C). Placental telomere length showed the least correlation with gestational age suggesting that telomere dependent senescence process is less important in placenta compared to fetal membranes.

3.2. Activation of stress associated p38MAPK[16] and cell cycle regulator/pro-senescence factor p53[11] in gestational tissues

Based on prior reports that p38MAPK induced senescence is associated with parturition in human pregnancy[8] and that p53 mediates senescence in mouse decidua [27], we examined the active forms of these cell cycle regulators in gestational tissues. As shown in Figure 2A, active (Phosphorylated) p38MAPK (P-p38 MAPK) was near undetectable in fetal membranes on day 10, but was seen on day 12 and demonstrated progressive increase until day 18. Placenta (Figure 2B) and decidua/uterus (Figure 2C) demonstrated activation of p38MAPK on day 12 and was present on all days until day 18. Total p38MAPK was constitutive from day 10 until Day 18. Total p38MAPK was seen at all gestational days in placenta and decidua/uterus with no obvious change in its expression. As shown graphically in Figures 2D-F, raw densitometric data of P-p38 /total p38 MAPK showed a ~ 1.5-fold increase on day 18 from day 10 whereas placenta (Figure 2E) and decidua/uterus (Figure 2F) had a moderate increase over the course of this period (~0.5 fold increase on day 18 compared to day 10). $\log_{10}$ transformed densitometric data from all three tissues showed a linear increase over time (Figures 2D–F).

Active p53 (P-p53) expression remained near the limits of detection in fetal membranes from day 10 and until day 17; however, a major spike occurred on day 18 (Figure 3A and D). Conversely, P-p53 was constitutively present in placenta (Figures 3B and E) and decidua/uterus (Figures 3C and F) from day 10 until day 18. Further, regression analysis of the $\log_{10}$ -transformed densitometry data (Figure 3D-F insets) suggests that there is an exponential association between the presence of P-p53 and gestational age in fetal membranes and placenta (3D and E, insets) while the data is insufficient to support such a hypothesis in decidua/uterus (3F, inset). This difference in the pattern of expression of active

p53 suggests a differential role for this antitumor/pro-apoptotic protein in mouse gestational tissues.

3.3. Immunofluorescent staining for p21, the downstream effector of both p38MAPK and p53, and cyclin dependent kinase inhibitor

To localize and determine any gestational age-related changes indicative of cell cycle arrest we performed analyses on tissues from days 10 and 18 of gestation. As shown in Figure 4A and 4B, staining intensity was higher on day 18 than day 10 in fetal membranes; however, data were inconclusive in placenta and decidua/uterus. Quantification of staining intensity was not performed.

3.4. Staining for SA- β Gal to determine senescence of gestational tissues

A potential effect of p38MAPK and or p53 activation is senescence. As shown in Figure 5, SA- β Gal positive (blue stained) cells were minimal on day 10 in fetal membrane tissues and increased gradually until day 18. In mouse placenta and decidua/uterus, the number of blue stained cells were very few on day 10 and 12, but were substantially higher from day 15–18. Therefore, fetal membrane senescence determined by the presence of SA- β Gal positive cells correlated well with telomere reduction, p38 and p53 activation and increased staining for p21.

4. Discussion

Here we utilize a mouse model to provide evidence that fetal membrane senescence occurs prior to labor and expulsion of the fetus during normal pregnancy. We have used adult females of average reproductive age under specific pathogen free conditions to document the longitudinal changes associated with senescence of intrauterine tissues, fetal membranes, placenta and decidua/uterus during mouse pregnancy. This study is not designed to determine the effects of aging on mouse parturition. We examined telomere length changes, stress associated p38MAPK and pro-senescence/cell cycle inhibitor p53 activation and histochemical evidence of senescence.

In summary, our data demonstrate the following in mouse feto-maternal gestational tissues; 1) telomere length is shortened in all gestational tissues as gestation progressed; however, telomere attrition was more highly correlated in mouse fetal membranes than any other gestational tissues. The lowest was seen in placenta 2) progressive activation of both p38MAPK occurs in all gestational tissues as gestation progressed but activation of both p38MAPK is dominant in fetal membranes. 3) progressive activation of p53 is seen in fetal membranes but constitutive presence of active p53 is seen in placenta and decidua/uterus throughout gestation. 4) Senescence of fetal membranes progresses throughout gestation whereas minimal placental and decidua/uterus senescence is detectable only towards the end of pregnancy. Senescence activation of all three intrauterine tissues is associated with gestational length in mouse pregnancy and is highest prior to normal delivery time (day 19). This study did not examine tissues after delivery as our goal is to identify senescence associated biomolecular and biochemical changes in tissues that are not confounded by labor associated conditions.

Our data are consistent with a report that extra-embryonic fetal tissues in mice exhibit decreased telomere length as normal gestation progresses and that differences in telomere dependent process that may signal parturition[4]. However, we now extend these findings to demonstrate that tissue specific senescence is not restricted to decidua [27]or placenta, but also occurs in fetal membranes. Further, we report that this process includes activation of p38 and p53, along with their downstream effector in cell cycle regulator p21, and generates histologic evidence of senescence. We hypothesize that the development of an aging phenotype ensues in mouse reproductive tissues as gestation progresses.

Unlike fetal membranes, P-p53 was seen constitutively in placenta and uterine and decidual tissues. This suggests that active p53 is a physiologic component of placental and uterine and decidual development during mouse gestation as a regulator of their growth, remodeling and development. Progesterone receptor driven deficiency in p53 generates senescence in decidual tissues immediately after implantation [27], and our data is consistent with this data as high levels of activated p53 are constitutively seen in decidua throughout gestation.

Existing data in mice also suggests that gestational length is primarily determined by maternal genotype[28], raising a question of the relative importance of ongoing processes related to maintenance of pregnancy and timing of parturition in fetal tissues. However, the function and longevity of fetal cells is not only dictated by maternal genotype, but the interaction between the environment provided in utero and the fetal genotype. We postulate that the changes in intrauterine environment (endocrine changes, for example, progesterone[29]) and mechanical factors (e.g. stretching as gestation progresses [21, 30, 31]), among others can potentially contribute to activation of stress associated p38MAPK. This then promotes the aging of the fetal tissues, thus preparing them for delivery. These interactions should be the focus of future studies.

We did not study SASP gene activation or inflammatory changes directly in this report. However, we used an existing dataset to report potential SASP activation along with senescence during mouse parturition. Previously, gene expression in the mouse decidua has been examined by microarray[26]. Examination of the fold-change expression of the genes suggested a set of consistently differentially expressing genes (up or down) with respect to the earliest time point (day 8.5). Using the model: **fold-change (from day 8.5) = $\beta_0 + \beta_1 \times \text{time point}$** , we calculated β_1 (the slope of the linear regression of fold-change as a function of time) and a consistency indicator, the fraction of fold-change differences that are increasing (or decreasing) from the previous time points. That is, if the consistency was calculated as 1, then the fold changes are all perfectly ordered; at 0.5 the fold changes alternate between increasing or decreasing. From this procedure, we generated a list of genes where β_1 was statistically different from 0 and consistency was at least 0.7. A PubMed search was performed using the headings “senescence OR cellular ageing OR senescence associated secretory phenotype (SASP [32])” to find consistently changing genes that are associated with senescence. Of 93 genes consistently increasing in their expression from day 8.5 till parturition, we found literature evidence that 12 were firmly associated with senescence (Table 1). Of sixty-five genes consistently decreasing in expression, we found 8 had evidence in the literature that a decrease in expression was related to senescence of cellular aging. Genes in the list not only include those that have been specifically identified

as SASP, but also those that are thought to be involved in oxidative stress, inflammation, or protection from these processes. We interpret this data to be supportive of the idea that SASP activation plays a role in parturition and that the decidua is poised to receive and transmit senescence signals from fetal membranes and other tissues. We specifically probed this data set for molecules that consistently increased or decreased their expression during gestation. However, there may be some SASP-associated molecules that exert their effects in discrete or peri-implantation time frames to “set the biologic and paracrine clock” for parturition. An example is interleukin 6 where deficiency has been linked to *prolonged* gestation[33] . It would therefore be of interest to explore the parturition phenotype of time (of gestation) or tissue (fetal membranes) restricted- models of interleukin 6 deficiency.

Taken together with the findings presented above, a cascade of senescence signals across several tissues, including fetal membranes and decidua, may be a profound driver of events leading to parturition. Moreover, our data places fetal membranes in the center of attention where membranes may act as signal callers of parturition as in humans. We report that increasing p38MAPK activation in all three compartments as gestation progress and this stress activated MAPK, a prosenescence activator, very well may be playing a major role in aging mechanisms in mouse intrauterine tissues. However, analyzing the data on fetal membranes, a classic difference between human and mouse parturition is noted. p38MAPK mediated senescence activation is mechanistically shown to be associated with transition of human not in labor membranes to labor associated inflammatory status (SASP). Similar increase in P-p38MAPK is also evident in mouse fetal membranes and it is associated with senescence related changes. However, p53 activation was not seen in human fetal membranes whereas mouse fetal membranes demonstrated progressive increase in p53 activation with highest levels on Day 18. This suggests a mechanistic difference in senescence activation between species, and suggests a synergy between p38 and p53 in determining senescence and delivery of fetus and fetal membranes in mouse models.

Besides playing a key role in human parturition fetal membrane senescence is a player in preterm premature rupture of membranes [34]. The recent observation that intrauterine injection of the senescence protein HMGB1 protein causes preterm birth in a mouse model leads us to assert that enhanced senescence may be a fundamental mechanism of adverse pregnancy outcome[23]. We moreover speculate that another source of enhanced senescence or inflammatory signaling might occur through abnormal regulation of the uterine microbiome [35, 36]. We assert that mouse models will be needed to determine the mechanisms relating senescence to early or abnormal parturition and to contribute further understanding of this process in humans.

Finally, our studies suggest the conjecture that fetal membranes and related tissue present a novel model for the examination of mechanisms of senescence and potentially a biomarker for future senescence-associated disease.

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Figure 1. Telomere length analysis of fetal membranes, placenta and decidua/uterus on different gestational ages

Shown are plots of telomere length (T/S Ratio) as a function of gestational age in days and the results of linear regression analysis, where Y is the value of T/S and X is the day of gestation. Negative values for the slope indicate gestational age dependent reduction in telomere length.

Figure 1A: Mouse fetal membrane telomere length analysis. Number of samples used for analysis: day 8 = 1; 9 = 2, day 10 = 10, day 12 = 3, day 15 = 10, day 17 = 4 and day 18 = 8. Linear regression: $Y = -0.1901 \cdot X + 4.193$, $R^2 = 0.5067$. Slope of line is significantly different from 0, $p < 0.0001$.

Figure 1B: Mouse placenta telomere length analysis. Number of samples used for analysis day 9 = 3, day 10 = 10, day 12 = 4, day 13 = 6, day 15 = 2, day 16 = 5, day 17 = 3 and day 18 = 7. Linear regression: $Y = -0.09000 \cdot X + 2.645$, $R^2 = 0.1501$. Slope of line is significantly different from 0, $p = 0.0135$.

Figure 1C: Mouse deciduas/uterus telomere length analysis. Number of samples used for analysis day 8 = 3, day 9 = 2, day 10 = 8, day 12 = 6, day 13 = 5, day 15 = 2, day 17 = 3 and day 18 = 8. Linear regression: $Y = -0.1317 \cdot X + 3.244$, $R^2 = 0.3305$. Slope of line is significantly different from 0, $p = 0.0003$.

Figure 2. Western blot analysis to determine the presence of Phospho (active) p38 mitogen activated protein kinase (MAPK) in fetal membranes, placenta and decidua/uterus on different gestational ages

Figure 2A and D: Western blot analysis of P-p38MAPK in fetal membranes: Progressive increase in P-p38MAPK was seen in fetal membranes starting at day 10 with maximum activation seen on day 18. **2D:** P-p38 /total p38 MAPK. Plot of the densitometric units observed after scanning of the relevant band as a function of gestational day. Inset, log₁₀-transformed data with R^2 and p value for testing hypothesis that the slope of the line generated is statistically significant from 0. Linear regression (not shown) of the non transformed data shows the slope of the line to be 0.1351, $R^2=0.8576$, $p=0.0239$.

Figure 2B and E: Western blot analysis of P-p38MAPK in placenta: Progressive increase in P-p38MAPK was seen in placenta starting at day 12 and with maximum activation seen on day 18. **2E:** Plot of the densitometric units observed after scanning of the relevant band as a function of gestational day. Inset, log₁₀-transformed data with R^2 and p value for testing hypothesis that the slope of the line generated is statistically significant from 0.

Figure 2C and F: Western blot analysis of P-p38MAPK in deciduas/uterus: Minimal activation of P-p38MAPK was seen on days 10 – 17 and maximum activation was seen on day 18. **2F:** Plot of the densitometric units observed after scanning of the relevant band as a function of gestational day. Inset, log₁₀-transformed data with R^2 and p value for testing hypothesis that the slope of the line generated is statistically significant from 0.

Figure 3. Western blot analysis to determine the presence of Phospho (active) p53 fetal membranes, placenta and decidua/uterus on different gestational ages

Figure 3A and C: Western blot analysis of P-p53 in fetal membranes (n=3 sets): Progressive increase in P-p53 was seen in fetal membranes starting at day 10 with maximum activation seen on day 18. **3D**: Plot of the densitometric units observed after scanning of the relevant band as a function of gestational day. Inset, log₁₀-transformed data with R² and p value for testing hypothesis that the slope of the line generated is statistically significant from 0.

Figure 3B and E: Western blot analysis of P-p53 in placenta (n=3 sets): P-p53 was constitutive in placenta on all 5 gestational days (10, 12 15, 17 and 18) tested **3E**: Plot of the densitometric units observed after scanning of the relevant band as a function of gestational day. Inset, log₁₀-transformed data with R² and p value for testing hypothesis that the slope of the line generated is statistically significant from 0.

Figure 3C and F: Western blot analysis of P-p53 in decidua/uterus (n=3 sets): P-p53 was constitutive in tissues on all 5 gestational days (10, 12 15, 17 and 18) tested **3F**: Plot of the densitometric units observed after scanning of the relevant band as a function of gestational day. Inset, log₁₀-transformed data with R² and p value for testing hypothesis that the slope of the line generated is statistically significant from 0. Neither the transformed (not shown) nor the transformed data showed a significant linear change with time.

Figure 4. Immunolocalization of p21 in fetal membranes, placenta, and deciduas/uterus from tissues isolated on days 10 and 18 of gestation

A: Fetal membranes on day 10 and 18 (n=3 sets): p21 (total and active) was localized in fetal membranes on day 10 and 18. Day 18 membranes had higher staining intensity than day 10.

B: Placenta on day 10 and 18 (n=3 sets): p21 (total and active) was localized in fetal membranes on day 10 and 18. Day 18 placenta had slightly higher staining intensity than day 10 placenta. **C: Decidua/uterus on day 10 and 18 (n=3 sets):** p21 (total and active) was localized in tissues on day 10 and 18. Based on staining intensities, no obvious differences in staining intensities were noted between day 10 or 18.

Figure 5. Determination of senescence in fetal membranes, placenta and decidua/uterus by staining for senescence associated β -Galactosidase (SA- β -Gal). Blue staining cells visualized under light microscope (20X images) on different gestational days are shown in 5A – 5C
 Figure 5A: SA- β -Gal staining of fetal membranes on day 10, 12, 15 and 18 (n=3 sets): SA- β -Gal positive cells were absent in fetal membranes on day 10 and appeared on day 12. Majority of cells were **SA- β -Gal** positive by day 15 and 18.
 Figure 5B: SA- β -Gal staining of placenta on day 10, 12, 15 and 18 (n=3 sets): SA- β -Gal positive cells were absent in placenta on day 10 and 12 appeared on day 15. Several of cells were **SA- β -Gal** positive by day 15 and 18.
 Figure 5C: SA- β -Gal staining of decidua/uterus on day 10, 12, 15 and 18 (n=3 sets): SA- β -Gal positive cells were absent in placenta on day 10 and 12. Several of cells were **SA- β -Gal** positive by day 15 and 18.

Table 1

Decidual genes and senescence.

Consistently up-regulated in decidua from day 8.5 onward				
$\beta 1^*$	gene	Common name	Relation to senescence or ageing	ref
7.468668102	Rbp4	retinol binding protein 4	Induces mitochondrial damage/oxidative stress	[37]
5.780080646	Chi313	chitinase 3-like 3	Induced by over-expression of IL-18 , ageing	[38]
5.410977925	Mmp12	Matrix metalloproteinase 12	Senescence associated secretory phenotype protein (SASP)	[11, 32]
3.742705807	Edn2	endothelin 2	Ageing and inflammation in diabetic retina	[39]
3.001408747	Chi314	chitinase 3-like 4	Ageing and ICAM expression	[39]
2.052888496	Pla2g7	phospholipase A2, group VII	Over expression leads to Reactive oxygen species,	[40]
1.701317444	Ccl6	chemokine (C-C motif) ligand 6	Induced by SASP protein Stem cell factor, in turn enhances eosinophil activation	[41]
1.404613666	Fn1	fibronectin 1	Senescence associated secretory phenotype protein (SASP)	[11, 32]
1.356156373	Flt1	FMS-like tyrosine kinase 1 (VEGFR1)	Senescence associated secretory phenotype protein (SASP)	[11, 32]
1.284768143	Cyr61	cysteine rich protein 61	Induced by oxidative stress and irradiation Increases IL-1 and MMP-1	[42–47]
1.253873973	Creg1	cellular repressor of E1A-stimulated genes 1	enhances p16(INK4a) -induced cellular senescence	[14]
Consistently down-regulated from day 8.5 downward				
$\beta 1$	gene	Common name	Relation to senescence or ageing	ref
−7.592696107	Mt4	metallothionein 4	Protection against oxidative stress	[48]
−5.379961452	Tdo2	tryptophan 2,3-dioxygenase	Protection against inflammation	[49, 50]
−3.535957009	Igf1	insulin-like growth factor 1	Inhibition leads to block in proliferation	[51]
−2.992145615	Ptn	pleiotrophin	Restoration of function in senescent vessels	[52]
−2.72952294	Rgs2	regulator of G-protein signaling 2	Rgs2 enhances transcription of the senescence inhibitor MKP7	[53]
−1.524795237	Pglyrp1	peptidoglycan recognition protein 1	Anti-inflammatory molecule associated with suppressing senescence	[54]
−1.422452384	Cfh	complement component factor h	down regulated in retinal pigment cells made senescent by oxidative stress	[55]
−1.149977719	Fst	follicle-stimulating hormone	decreased in senescent hair follicles	[56]

* slope of the line fold change (from day 8.5) = $\beta 0 + \beta 1^* \text{ time over day 8.5}$. All slopes noted are significant. Negative values indicate decreasing expression over time. Higher absolute values indicate a more aggressive change with time.