Gene expression in the twilight of death

The increase of thousands of transcripts has implications to transplantation, cancer, and forensic research

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After a vertebrate dies, many of its organ systems, tissues, and cells remain functional while its body no longer works as a whole. We define this state as the ''twilight of death'' – the transition from a living body to a decomposed corpse. We claim that the study of the twilight of death is important to ethical, legal and medical science. We examined gene expression at the twilight of death in the zebrafish and mouse reaching the conclusion that apparently thousands of transcripts significantly increase in abundance from life to several hours/days postmortem relative to live controls. Transcript dynamics of different genes provided ''proof-of-principle'' that models accurately predict an individual's elapsed-time-of-death (i.e. postmortem interval). While many transcripts were associated with survival and stress compensation, others were associated with epigenetic factors, developmental control, and cancer. Future studies are needed to determine whether the high incidence of cancer in transplant recipients is due to the postmortem processes in donor organs.

Keywords:
DNA microarrays; Gene Mmeter; postmortem gene expression; stress

Introduction

A continuing enigma of life is what happens to complex biological systems that are stressed. The body of an adult vertebrate, as an example, consists of many organ systems, tissues, and cells that work together to maintain homeostasis – and thus, sustain "life" of the whole. The questions we were interested in are: *what happens to the "whole" when a body is subjected to extreme stress, such as sudden death? Do organ systems, tissues and cells simply shutdown or is there a gradual cessation, with some parts of the body attempting to re-establish homeostasis?* Surprisingly, there is a paucity of information to address these questions and no baseline data for comparing less severe forms of stress (e.g. disease, old age). To open the door on the biology of death, we examined how gene expression changes with increasing postmortem time.

In today's world, the definition of death and the time of a person's death have ethical, legal, and medical implications – for example, *what is the optimal time for procuring organs for transplantation from a donor?* Only in recent human history has the exact time of a person's death been considered. In the past, a person was either alive or dead – the gray areas between the two states did not exist. We argue that understanding the biology of death is needed to interpret the gray areas. To frame our essay, we introduce two definitions, organismal death, and "twilight of death"; present the ethical, legal, and medical implications; discuss the methodology used in our recent studies [2, 3]; and overview the most important findings we discovered.

Definitions

There are many definitions of death such as denouement death, threshold death, and integration death [1]. A unifying theme of the definitions is that death is a process. The "denouement death" definition is closest to being absolute: the process of death is complete when the last physiological processes maintaining homeostasis cease to function. Yet, this definition does not consider the gray areas between life and death. For example, *is a person considered alive when the penultimate physiological process ceases to function?* "Threshold death" is defined as the point where some life remains but there

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Abbreviations:
COMM, copper metabolism; **COUP,** chicken ovalbumin upstream promoter; **NGS,** next-generation-sequencing.

is no way of preventing loss of life. "Integration death" is defined as the point when the body and its various physiological functions cease to function as a whole. Since we were not completely satisfied with these definitions, we defined "organismal death" in our study as what happens when an adult suddenly dies, for example in a heart attack or after a cervical fracture (i.e. broken neck). Cells in the body may be alive but the body does not function as a whole. Hence, our definition combines both aspects of threshold and integration death into one.

The "twilight of death" is a term defining the gray areas between life and organismal death. We introduce this term because there was no previous definition in the scientific literature. Our rationale is that minutes after organismal death, there is no reason to suspect all bodily and cellular functions will suddenly cease. On the other hand, we know that within hours to days, a body will eventually decompose by natural processes. Hence, the "twilight of death" represents the window of time between organismal death and the start of decomposition – a time when bodily and cellular functions continue but not all cells are dead yet. In reality, little is known about what happens in terms of the biology in this window of time, and that is what compelled us to study it.

Ethical implications

Research on the "twilight of death" is important to the medical community because the very concept of death and the putative grounds for diagnosing it remain controversial [4]. Take, for example, the position that organ donors must be dead for vital organ removal – *what does "dead" really mean in light of the definitions provided above?* An ethical dilemma could arise if a physician waits until a donor's denouement death because the relative health of the donor's organ might be compromised (e.g. increased ischemia time reduces transplantation success [5]) and the transplant recipient might die as a consequence. On the other hand, the hasty retrieval of the organ from a donor – who might not be completely "dead" – could lead to death by organ donation, which obviously violates a physician's ethical imperative to "first do no harm." While Potts and Evans [6] argue that there is no firm scientific or philosophical basis for the procurement of organs for transplantation, studies of the biology in the twilight of death might yield a better understanding of the science of death, and perhaps yield an improved distinction between what constitutes life and death.

Legal implications

The time of a person's death (commonly known as postmortem interval or PMI) is important to civil and criminal investigations because it can help solve mysteries. In civil cases, such as those involving life insurance fraud, for example, investigators need to know the time of death in order to ascertain whether the person was alive or not when the policy was in effect (B. Anderson, Five notorious, homicidal tales of life insurance fraud Real-life clients from hell. LifeHealthPro. http://www.lifehealthpro.com/2013/10/25/5-notorious-homicidal-tales-of-life-insurance-frau, Accessed January 3, 2017). In criminal investigations involving death or murder, it is essential to know the time of death because often there are no witnesses and it is difficult to determine the association between a criminal and the victim. In murder cases, the time of death can help investigators eliminate certain people from the suspect list and therefore focus on others, which speeds up the investigative process. By far, accurate estimation of the time of death is considered to be the most important and most complex tasked performed by forensic investigators [7]. A method that accurately determines the time of a person's death is highly desired because current methods yield variable results [8].

Medical implications

Organs for solid organ transplantation are mostly procured from brain dead donors [9]. By definition, "brain dead" means that the donor has irreversible loss of brain functions [10]. Organs procured from these donors are regarded as suboptimal in terms of quality because brain death causes a massive inflammatory response that triggers substantial circulatory, hormonal, and metabolic changes in the donor's body [11, 12]. These changes affect organ quality leading to potential immunogenicity problems and risk of organ dysfunction in transplant recipients [13]. Several strategies have been implemented to assess and improve upon the quality of organs for transplantation: for example developing donor risk scores and conducting baseline biopsy measurements [14], modulating the nutrition of the donor prior to organ procurement [13, 15], and preserving the organs using various machine perfusion methods [16–18]. The focus of recent studies has been optimizing preservation methods to ameliorate damage and restore organ function. The preservation methods are suppose to protect against ischemic injury, recondition the organ before reperfusion, and/or maintain physiology [19].

The success/failure of these methods have been determined by monitoring gene expression of oxidative stress, apoptosis, adhesion, and inflammation biomarkers [20]. Yet to date, gene expression studies have not been conducted to investigate whether the elapsed-time-since-death (i.e. the postmortem time) of the donor is an important factor effecting organ quality and/or transplantation success. Postmortem time has a significant effect on gene expression because physiological changes take place in the body after organismal death (e.g. hypoxia, pH). Hence, it is reasonable to suggest that postmortem time of the donor affects organ quality and that examining the relationship between gene expression and postmortem time (prior to preserving an organ) might yield valuable baseline information on organ quality that has previously not been considered.

Methodology for studying the twilight of death

In life, genes play a central role in responding to the physiological demands of the cell by producing transcripts (mRNAs). The "turning on" of genes and the production of mRNAs report the inner workings of genetic regulatory networks – for example, what the cells need to survive, metabolize and proliferate. In the twilight of

death, we would expect some of the same genes operating in life to be "turned on" because many cells are not aware of the organism's death. With increasing postmortem time, however, the transcripts of response genes associated with hypoxia and stress compensation are expected to increase in abundance because the heart is no longer supplying the cells with oxygen and the cells become hypoxic. In addition, transcripts of immunity genes are expected to increase in abundance because they play roles in preventing microbial growth.

We induced organismal death in zebrafish by immersing them into ice-cold water and in mice by cervical dislocation (i.e. breaking their necks). Messenger RNAs were collected from homogenates of the whole zebrafish and the brains and livers of mice at several postmortem times. The change in gene transcript abundances with postmortem time was determined using a high throughput approach.

Several high throughput approaches could be used to measure transcript abundances: conventional DNA microarrays and next-generation-sequencing (NGS). To facilitate sample comparisons, these approaches require the data to be normalized, which introduce biases that can significantly affect interpretation of the data [21, 22]. To avoid these biases, we developed and used the "Gene Meter" approach, which precisely determines the transcript abundances of specific genes in biological samples and minimizes noise in the microarray signal [23–26]. The reason this approach is precise is because the behavior of every microarray probe is determined by calibration – which is analogous to calibrating a pH meter with buffers. Without calibration, the precision and accuracy of a meter is not known, and one cannot know how well the experimental data fits to the calibration (i.e. R^2). The advantage of the Gene Meter approach over conventional DNA microarray approaches is that the calibration takes into consideration the non-linearity of the microarray signal. Furthermore, normalization of the calibrated probes is not required to compare biological samples. It should be noted that we recently demonstrated that the same approach could be used for NGS [3].

Many gene transcripts increase in abundance in the twilight of death

Thousands of postmortem gene transcription profiles from 44 zebrafish (*Danio rerio*) and 20 house mice (*Mus musculus*) [2] were produced using the Gene Meter approach. Approximately 99% of gene transcripts decreased in abundance within 30 min of organismal death and continued to decrease with postmortem time. The decrease in transcript abundances presumably reflects degradation and/or the down-regulation of genes. Yet, about 1% of the gene transcripts (548 in the zebrafish and 515 in the mouse) significantly increased in abundance (relative to flash-frozen live controls) for up to 48–96 h postmortem. The diversity of gene transcripts (with increased abundance) declined after 24 h postmortem in both animals, presumably indicating a natural threshold was reached.

Interestingly, there were qualitative and quantitative differences in degradation profiles and the gene transcripts between the two mouse organs (liver and brain) [2]. Specifically, mRNA increased in the first hour and then gradually decreased in the mouse brain, while mRNA gradually decreased with postmortem time in the mouse liver (see Fig. 2 in [2]). These findings are aligned with previous postmortem studies showing that mRNAs are more stable in the brain than the liver [27]. We also found fewer transcript profiles significantly increased with postmortem time in the liver ($n = 35$) than in the brain ($n = 476$). Taken together, it appears that there are significant differences in the number of genes that had increased transcript abundances between organs, which warrants further study.

Variability in randomly selected transcript profiles of the 1% is shown in Fig. 1. Each datum point represents the mRNA collected from an individual mouse homogenate, which essentially is a complex mixture of many different types of cells/tissues from the same organ. The transcript abundance of the *Chicken ovalbumin upstream promoter (COUP) transcription factor 1* gene increased after organismal death to reach a maximum at 1 h postmortem; the *NULL* gene (no available annotation) reached maxima at 1 to 6 h; the *Copper metabolism (COMM)-domain containing protein* gene reached maxima at 12–24 h; and the *Zinc-finger protein 652 (Zfp652)* gene gradually increased with time to reach a maximum abundance at 48 h postmortem. Figure 1 shows that replicated samples of the same gene transcript taken at the same postmortem time are highly similar, indicating high reproducibility of sample replicates in their response to organismal death.

In terms of function, the *COUP transcript factor 1* gene plays important roles in development and differentiation [28], the *COMM-domain containing protein* gene encodes a factor promoting ubiquitination of target proteins [29], and the *Zfp652* gene is involved in tumor suppression [30]. While there may appear to be interesting patterns in the transcript profiles and their corresponding functions in Fig. 1, no definitive relationships were found in hundreds of transcript dynamics and functions examined in our original studies [2, 3].

As shown in Fig. 1, the transcript abundances of genes yielded different dynamics. When the transcription profiles of hundreds of genes was examined using principal component analysis, we discovered that the timing of the increased abundances, the peak abundances, and the durations occurred at different times for different genes, indicating apparent order in the twilight of death. These results are aligned with the idea that death is a process that follows a chronological pattern.

While the most obvious explanation for the increases in gene transcript abundances over the controls is that the synthesis rate of mRNAs far exceeded that of the degradation rate [31], there are other plausible reasons. For example, the increased transcript abundances could be attributed to enrichment of stable non-degrading RNA in the transcription pool that changed with postmortem time [32]. However, we ruled this out in our study [2] because total RNA concentrations of the mouse samples remained the same for the 48 h sampling period. It is also plausible that the increased abundances could be due to differential stability of cells types with postmortem time [33]. In other words, some cells are

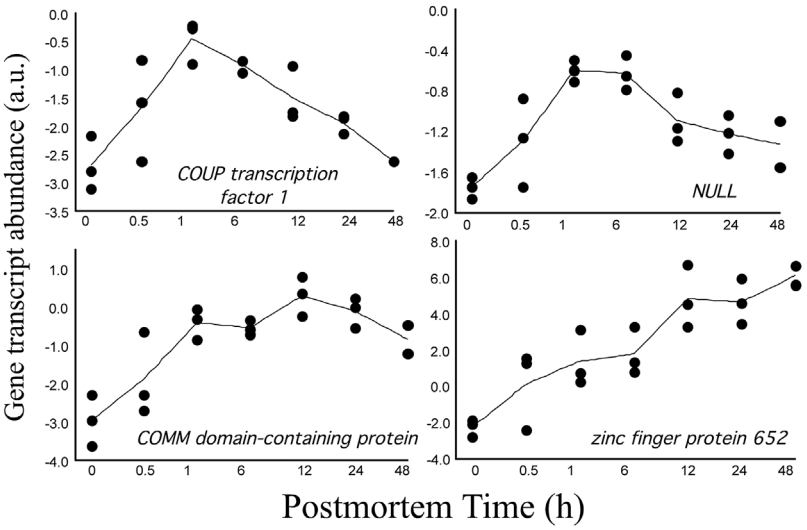

Figure 1. Transcriptional profiles of four mouse brain genes. Each circle represents the datum point of the mRNA transcript from one individual mouse. Null, no available gene annotation. Gene transcript abundances are log2 arbitrary units (a.u.).

more resilient than others and are the last ones to die. Different cell types have different transcript abundances and therefore the observed abundances could represent the changing of transcript pools by cell type. It is important to recognize that the transcript pool from a mouse sample is a homogenate of mRNAs from many different tissues and cell types in the same organ and it is certainly possible that it changed with postmortem time.

PMI can be predicted from transcript abundances of a set of genes

Given the low variability of the triplicate samples and differences in the transcript dynamics for different genes, we rationalized that this information might be useful for determining the elapsed-time-since-death (i.e. PMI) of an individual. In other words, by capturing the transcript abundances of a set of genes of a corpse and knowing the corresponding coefficients for a set of genes, we could determine how long a corpse has been dead (i.e. organismal death [3]).

For the liver (Fig. 2, left panel), the R^2s of the testing and validation data (not used in training) were 0.99 and 0.97, respectively. The combined R^2 of the training, testing and validation data was 0.98 with a slope of 0.97. For the brain sample (Fig. 2, right panel), the R^2s of the testing and validation data were 0.95 and 0.87, respectively. The combined R^2 of the training, testing, and validation data was 0.95 with a slope of 0.87. These results show that transcript abundances of a set of genes can provide reasonably accurate predictions of the PMI.

Our experimental design provided "proof of principle" and did not consider factors such as temperature, which have been considered in other PMI models (e.g. [34]). Future studies are needed to incorporate the effects of temperature and the environment on PMI predictions in order to make the design more universal.

With regard to organ transplantation, the same experimental design could be used for: (i) assessing the quality of organs in human donors prior to transplantation; and (ii) evaluating the effectiveness of various preservation methods to ameliorate damage and organ function due to prolonged storage time.

Transcripts of survival and stress compensation genes increase in abundance

We found that transcripts from stress response genes significantly increased in abundance with postmortem time [2]. Most notable stress genes included those encoding heat shock (e.g. *Hsp*), hypoxia-related (e.g. *Hif1ab*) and oxidative stress (e.g. *Gadd45a*, *March4*) proteins. Transcripts from innate (e.g. *Laao*, *Tox2*) and adaptive (e.g. *Ms4a17.a1*, *Usp18*) immunity genes were also found at increased abundances, which make sense since vertebrates have evolved ways to protect the host against infection and injury in life [35]. We found increased abundance of transcripts of apoptotic genes (e.g. *Fosb*, *Bcl2l11*), which were anticipated, as apoptotic processes are involved in killing damaged cells for the benefit of the organism as a whole. We also found increased abundance of transcripts of transport genes (e.g. *Aralar2*, *Abcc5*), which presumably are responding to the stress by maintaining ion/solute/protein homeostasis, and controlling the influx/efflux of carbohydrates, proteins, signaling molecules, and nucleic acids across membranes. Taken together, transcripts of these genes increase in abundance presumably to cope with perturbations, reestablish homeostasis, and stabilize the cytoskeleton.

Transcripts of developmental control and cancer-associated increase in abundance

An unexpected finding of our study [2] was the increased abundances of developmental control transcripts (e.g. *Mdga2*, *Ripply3*) with postmortem time. In life, these genes are mostly involved in regulating developmental processes from early embryo to adult; and therefore, not anticipated to increase in abundance in the twilight of death or in response to stress. The increased abundances suggest that the genes are no longer silenced. It is possible that the nucleosomes are unwound by histone modification and chromatin interactions, which enables transcription factors and RNA polymerases to transcribe the previously silenced genes. Support for this idea comes from the concomitant increases of epigenetic regulatory transcripts (e.g. *Hist1h3f*, *Yeats2*) in the twilight of death [2]. Moreover, it has been previously shown that epigenetic

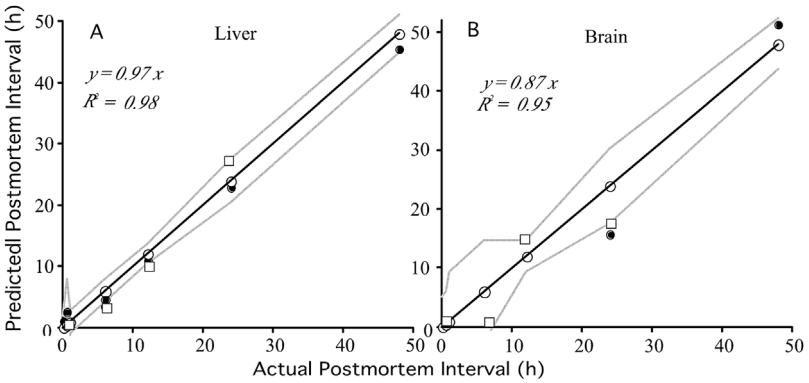

Figure 2. Predicted versus actual PMI determined for the mouse liver and brain. R2 and slopes are based on both training and testing datasets. Gray line represents 99% confidence limits of the linear regression. Open circles, training data; closed circles, testing data; open squares, validation data (not used in training).

factors hypermethylating the chromatin increase expression of developmental genes in cancer [36].

Several medical studies have reported increases in developmental control transcripts in response to stress such as injury of human knee articular cartilage [37], atherosclerotic-associated diseases [38], and lung cancer [39]. It is therefore, reasonable to suggest that increased abundance of developmental control and cancer-associated transcripts might reflect a universal response to stress that has not been previously recognized.

Is postmortem gene expression dysregulation in solid organs a contributing factor to oncogenesis in transplant recipients?

It is well established that solid organ transplant recipients have a higher risk of developing cancer than the general population [40, 41]. Specifically, cancer accounts for approximately 10–30% of deaths in transplant recipients [42]. It is generally assumed that the higher risk is due to prolonged immune suppression to prevent rejection of the donated organ following transplantation [43]. Although a rare occurrence (~2%), cancer can originate from pre-existing tumors in the donor organ [44]. We found an increased abundance of many cancer-associated (tumor suppressor [e.g. *Tnfrsf9*, *Ell*] and oncogenic [e.g. *Tpr*, *Csnk2a1*]) transcripts in the twilight of death in both organisms (zebrafish and mouse) [2]. Hence, we propose that postmortem gene expression dysregulation might be a contributing factor to oncogenesis in transplant recipients since postmortem time of the donor (i.e. how long the donor was dead before organ procurement) might increase the cancer risk as the diversity of cancer-associated gene transcripts was found to drastically increase in the first 24 h of postmortem time [2]. We conjecture that it might be possible to reduce this risk by pre-treating the organ with targeted oncogene inhibitors as a part of the transplant conditioning.

What does the increased transcript abundances tell us about stressed biological systems?

Since increased transcript abundances occurred in both adult model vertebrates (i.e. zebrafish and mouse) [2], it is reasonable to suggest that other multicellular eukaryotes will display a similar phenomenon. This begs the question: *What does this mean in the context of organismal life?* As adults, vertebrates represent highly ordered structures – evolved and refined through natural selection and self-organizing processes [45]. Under extreme stress, these ordered structures undergo a thermodynamically driven process of spontaneous disintegration, with most pathways (99% of genes) becoming nonfunctional, while others (i.e. 1% of genes) remaining functional (at least for some time). Hence, the increased abundance of specific transcripts could be attributed to components of resilient pathways (e.g. stress compensation and survival genes) being less affected by stress than others. In terms of organismal life, evolution might have played a role in pre-patterning of these pathways, but it plays no role in their disintegration fate. One could argue that some of these pathways have evolved to favor healing or "resuscitation" after severe injury, which would be a possible adaptive advantage. Future studies examining postmortem transcript profiles of other animals (e.g. humans, *Hydra*, earthworms) might reveal whether this phenomenon is a universal response to stress.

It is possible that some genes (i.e. the 1%) are transcribed when they overcome thermodynamic and kinetic barriers in the twilight of death. Consider repressor proteins, for example, that in life bind promoter regions of genes preventing their transcription. In the twilight of death, these repressor proteins could be degraded, leading to upregulation of the previously repressed genes. The barrier in this example is the repressor proteins. Several other examples of thermodynamic and kinetic barriers include: (i) the tightly wrapped nucleosomes that unwind, allowing access of molecular transcription machinery to developmental control genes; (ii) the nucleopores that allow the exchange of mRNA and other molecules between the mitochondria and the cytosol; and (iii) the ion/solute protein channels that control intracellular ions regulating apoptotic pathways [2]. Taken together, it is possible that genes are transcribed simply because energy barriers (maintained in life) are breached in stressed situations.

Conclusions and outlook: Impact and future of postmortem research

Before embarking on the study of transcript dynamics in organismal death, we had no idea that the "twilight

of death" would be so full of surprises. Our study has gleaned new insights into the potential ramifications of organ transplantation, discovered a new way to determine the PMI using transcript profiles, and provided a new perspective on organismal death: gene expression apparently continues in the twilight of death. The gray areas between life and death appear to be more complicated than previously thought as demonstrated in the transcriptional profiles that vary in the timing of their increased abundances, their peak abundances, and their durations. There is order in the twilight of death as shown by the low variability in transcript abundances of replicated samples (representing individual organisms) with postmortem time. The surprising finding here is that there seems to be common pathways to denouement death. If we arrested these pathways by providing nutrients and oxygen to organ tissues, what would happen? Is it possible for cells to revert back to life or differentiate into something new or lose differentiation altogether, such as in cancer? We speculate that the recovering cells will likely depend on the postmortem time – at least when potentially interesting effects might be seen. The take home message here is: a lot can be learned about life by studying the twilight of death.

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