Effect of high D-galactose concentrations on the proliferation and viability of endothelial cells.

Hannah Goddard, Elissa Ony, Benjamin Sinclair

Department of Biological Sciences, Thompson Rivers University

Cell Physiology Laboratory BIOL 3520_L01

Supervised by Dr. Joanna Urban

Abstract:

Endothelial cell cultures provide a model for investigating cellular responses to metabolic stress. This study examined the effects of D-galactose on the proliferation and viability of Ea.hy926 endothelial cells. The aim was to determine whether varying levels of galactose influence cell growth, survival, and metabolic activity. Cells were incubated in and fed media containing various concentrations of D-galactose and their proliferation and viability were assessed using the BCA protein assay and direct cell counting, respectively. Results demonstrated a clear, dose-dependent decrease in cell viability with increasing galactose concentration. However, cell proliferation did not follow this trend. Potential sources of experimental error include galactose precipitate formation within the cell cultures, and experimental data points near the limits of the linear region for the BCA assay standard curve. Future research will aim to increase experimental robustness and accuracy by incorporating higher galactose concentration treatments and replicating trials across triplicate days.

Introduction:

The culturing of mammalian endothelial cells is a common practice in research, clinical, and pharmaceutical settings. Adherent cells are often cultured due to convenience of visual inspection using an inverted microscope (Urban, 2025). Usually, cells which have unlimited potential to divide (immortalized cells) are chosen for culture, although important experiments can be performed on a more relevant cell line. There are a multitude of techniques to achieve successful cultures, including varying media and incubation conditions. For instance, cells can be cultured with a constant atmospheric CO_2 concentration, or without. In general, a medium should contain a carbon source, amino acids, salts, buffering agents, and other compounds. A medium can be supplemented with fetal bovine serum (FBS) which contains growth factors and proteins (Urban, 2025).

In some media, D-glucose is replaced with D-galactose as a carbon and energy source. Although less efficient than glucose, galactose is oxidized anaerobically by glycolysis through the intermediate glucose 6-phosphate (Nelson and Cox, 2017). Galactose preferentially enters the oxidative phosphorylation pathway to produce energy in the form of ATP. Therefore, when galactose is the primary energy source, the cell is more reliant on aerobic respiration. Additionally, lactate production through anaerobic fermentation is minimized (Aguer et al., 2011). This is favourable when growing a culture in a CO_2 free environment, because CO_2 is used by cells to maintain physiological pH, and high lactate concentrations lower pH (Sigma Aldrich, 2007).

Leibovitz's L-15 media is a commercially available media containing D-galactose as the primary carbon source, which was originally formulated for use in CO_2-free systems (Sigma Aldrich, 2007). This experiment uses complete media consisting of Leibovitz's L-15 media supplemented with 10% FBS and 0.6% antibiotic/antimyotic solution. Cells were cultured in a 37 ^oC incubator at ambient (uncontrolled) CO_2 conditions. Complete media with varying concentrations D-galactose, 5 mmol/L, 25 mmol/L, and 50 mmol/L, were prepared to determine the effect of high galactose concentrations on cultured Ea.hy926 cells, a hybrid cell line. It is an adherent and immortalized cell line formed from fusion of human umbilical vein, endothelial cells, and lung carcinoma cells (Lisec et al., 2024). The present study aimed to investigate the effect of high galactose concentrations in media on the proliferation and growth of endothelial cells. It is hypothesized that increased galactose concentration will upregulate metabolic

enzymes (especially mitochondrial) which will be shown by total protein content increase. Cultures with high galactose may also show evidence of lower viability due to cytotoxicity. A bicinchoninic acid (BCA) assay was performed to determine total protein content and live cells were counted using a hemocytometer and microscope to determine cell viability.

Materials and Methods:

Media preparation:

To determine the effect of D-galactose on endothelial cells, 300 mL of Leibovitz's L-15 media with 10 % FBS and 1 % antibiotic/antimycotic solution was prepared. A 0.2 µm syringe filter was used to transfer and sterilize 100 mL aliquots to 3 autoclaved bottles. Appropriate amounts of solid D-galactose were dissolved in Milli-Q water and added aseptically, using a 0.2 syringe filter, to complete media to create 25 mmol/L and 50 mmol/L culture media. Our treatment concentrations were chosen based on previous experiments in literature (Zhang et al., 2020; Gao et al., 2021; Yorek et al., 1991). Leibovitz's L-15 media already contained 5 mmol/L D-galactose so that was used as a positive control treatment without any additional galactose. All media was stored in the refrigerator for future use.

Preparation of cell cultures:

Ea.hy926 endothelial cell cultures were subcultured and split evenly into four T75 flasks. All cultures were incubated at 37 °C with uncontrolled (ambient) CO_2 conditions and fed twice a week. After 1 week incubation one culture was fed with 50 mmol/L galactose medium, one was fed with 25 mmol/L galactose medium, and two were kept as 5 mmol/L galactose controls. All cultures were incubated for two weeks at 37 °C with uncontrolled CO_2 conditions and fed their respective media twice per week. Figure 1 below shows what the high galactose treatment cultures looked like after one week of incubation.

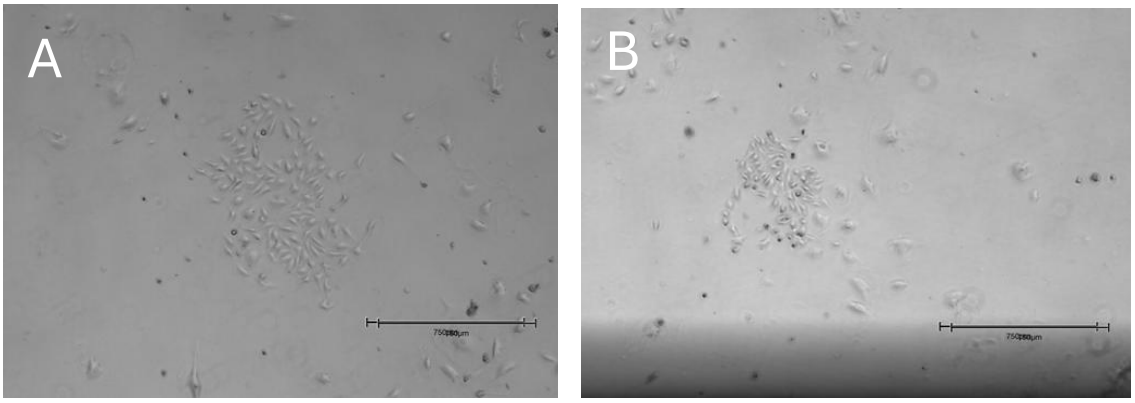

Figure 1. Experimental cell cultures exposed to 25 mmol/L (A) or 50 mmol/L (B) D-galactose visualized using inverted microscope.

Scratch wound assay:

A 24 well plate was seeded for a scratch assay using a control culture. However, after 1 week of incubation at 37 °C with uncontrolled CO_2 conditions, confluent growth was not attained. Due to time constraints the scratch wound assay was abandoned and not discussed further.

Cell viability test:

To test cell viability when endothelial cells were exposed to high concentrations of D-galactose live cells were counted using trypan blue dye and a hemocytometer. This was done by first following steps 1 to 8 of the subculture procedure provided in the BIOL 3520 student lab manual (Johanna, 2025). Once the cell pellet was obtained from centrifugation, it was resuspended in 1 mL of PBS solution. In a microcentrifuge tube, 20 µL of the resuspension was mixed with 20 µL of trypan blue dye, and live cells were counted using a hemocytometer and an inverted microscope. Two replicate counts were done for each experimental treatment and the control treatment. The two replicates were averaged, and standard deviation was calculated. The number of live cells/mL was calculated using the equation below.

$$\text{Cells/mL} = \frac{Total\ counted\ cells}{5\ squares} \times 2 \times 10\,000$$

BCA protein content assay:

The bicinchoninic acid (BCA) assay was used to determine cell proliferation based on protein content. The rest of the resuspensions, not used for cell counting, were mixed with RIPA lysis buffer to release total protein into solution. Steps 1 to 5 of the procedure for animal cell lysis after sub-culturing (pellets) were followed according to the student lab manual (Johanna, 2025). Samples were prepared from an 8:1 ratio of lysate:BCA working reagent and incubated at 60 °C for 5 minutes. Absorbances for samples and BSA standard solutions, whose concentrations are below (Table 1), were read in triplicate using the nanodrop instrument (Figure 2) at 562 nm. All data are presented as averages ± the standard deviation of the mean where *n* indicates the number of replicate measurements. Confidence intervals were also calculated at a 95 % confidence level using Student t-test values.

Table 1. Standard solutions and their protein content used for BCA protein content assay.

Standard	Protein content (mg/mL)
0	0.00
1	0.25
2	0.50
3	0.75
4	1.00

Figure 2: Nanodrop instrument used to measure absorbance at 562nm.

Results:

Cell viability test:

As D-galactose concentration in the culture media increased from the control group 5 mmol/L to the x10 treatment, 50 mmol/L, the number of live cells/mL decreased significantly (Figure 3). The control treatment, 5 mmol/L D-galactose, had the highest live cell count with 130,000 ± 14142.14 live cells/mL. As galactose concentration increased to 25 mmol/L, the live cell count decreased to 66,000 ± 2828.43 live cells/mL. The highest galactose concentration treatment, 50 mmol/L, lowered cell count even further to 16,000 ± 11,313.71 live cells/mL. These results indicated cell viability decreased as the concentration of D-galactose increased. All experimental live cell counts were found to be considerably lower when compared to cell counts from cultures earlier in the semester.

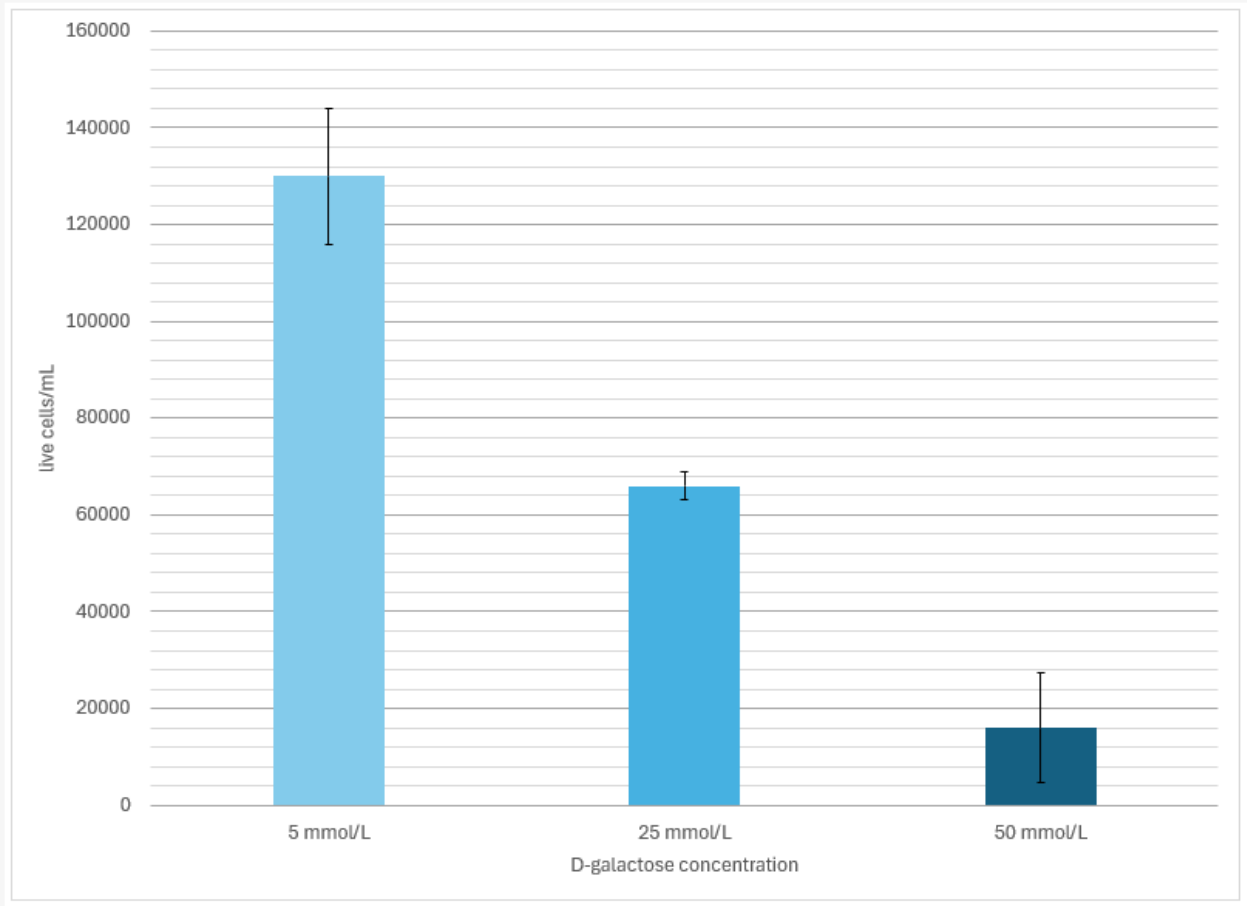

Figure 3. Live cells/mL counted using hemocytometer for Ea.hy926 endothelial cell cultures exposed to various concentrations of D-galactose in culture medium (n = 2).

BCA protein content assay:

A standard curve of average absorbance (562 nm) values as protein content increased was produced (Figure 4). The highest concentrated standard, 1.00 mg/mL protein content, was an outlier and not close to our unknown samples, therefore it was not included in the standard curve. The standard curve resulted in an R squared value of 0.9833 and an equation of the line of y = 1.56x + 0.055, used to calculate protein content in samples. After 14 days of incubation at 37 °C the 25 mmol/L galactose sample unexpectedly had the largest average protein content of 0.129 ± 0.003 mg/mL with a 95 % confidence interval of 0.120 mg/mL to 0.138 mg/mL (Table 2). The 5 mmol/L galactose sample was found to have the second largest protein content, 0.078 ± 0.007 mg/mL with a 95 % confidence interval of 0.060 mg/mL to 0.096 mg/mL (Table 2). Lastly, the sample exposed to the highest galactose concentration, 50 mmol/L was found to have the lowest protein content, 0.022 ± 4.25 $x10^{-17}$ mg/mL with a 95 % confidence interval of 0.022 mg/mL to 0.022 mg/mL (Table 2). Despite standard deviations being low, these values may not be accurate due to experimental absorbance values falling on the outskirts of the linear range of the standard curve (Figure 4).

Table 2. Average protein content in mg/mL (n = 3) of experimental cultures exposed to different concentrations of D-galactose in culture medium and calculated confidence intervals with a 95 % confidence level.

D-galactose concentration in media (mmol/L)	Average protein content (mg/mL)	95 % confidence interval
5	0.078 ± 0.007	0.120 - 0.138
25	0.129 ± 0.003	0.060 - 0.096
50	0.022 ± 4.25 $x10^{-17}$	0.022 - 0.22

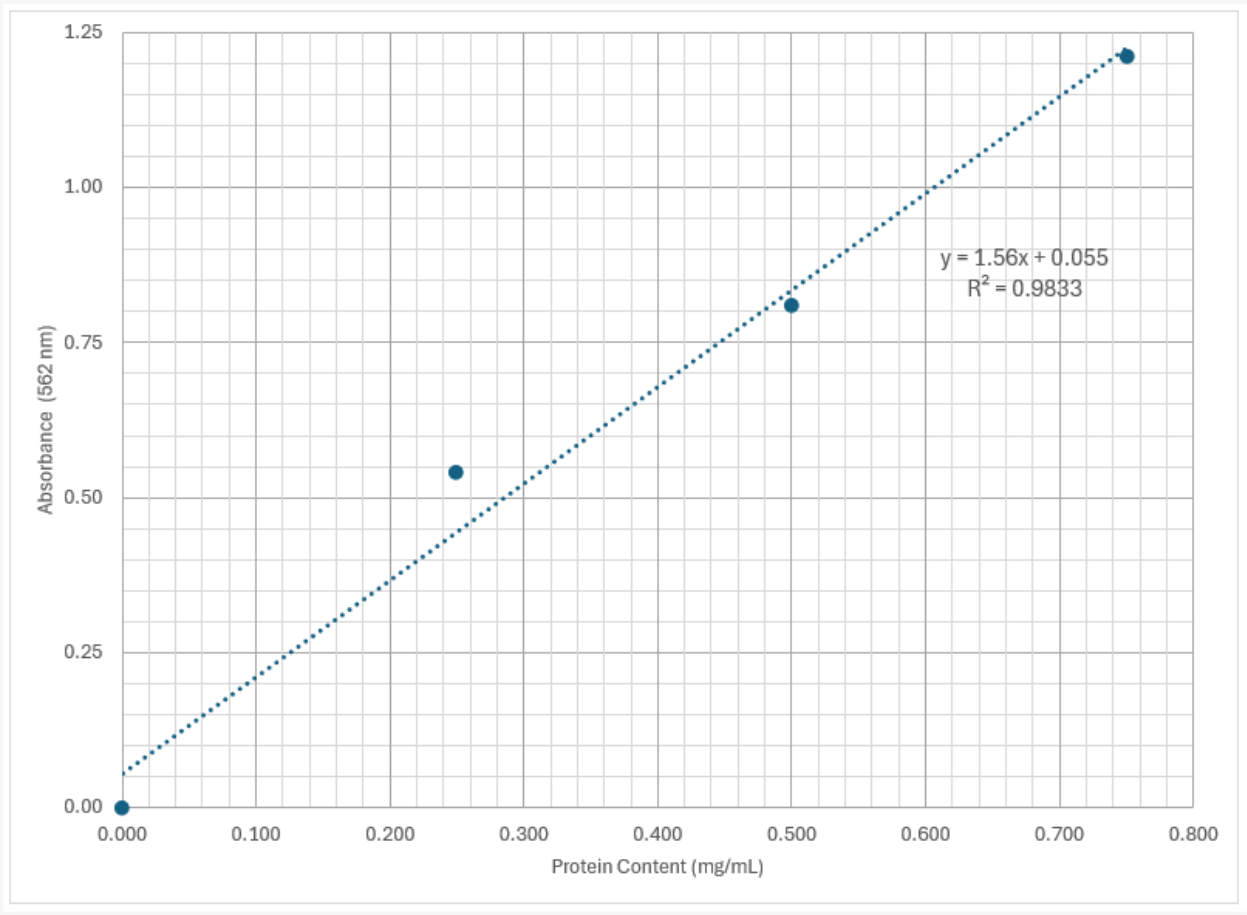

Figure 4. BCA assay standard curve displaying protein content in mg/mL as average absorbance at 562 nm increases (n=3).

Discussion:

Summary:

This experiment investigated the effect of high D-galactose concentrations on the growth of cultured endothelial cells by monitoring cell proliferation and cell viability. A live cell count and BCA total protein content assay were both performed. Cell viability decrease as media D-galactose concentration increased was observed, as determined by live cell counting. Unexpectedly, the moderate galactose concentration of 25 mM showed the highest protein content of the three treatments, while the 50 mM treatment showed the lowest. Since our experiment had minimal treatments, replicates and assays, it is hard to draw meaningful conclusions from our results. However, our results could be evidence that increased galactose induces higher metabolic protein expression levels, but at high enough concentration, galactose shows cytotoxicity.

Two main reasons for acute toxicity of galactose are suggested here. The first is the sterile addition of galactose to pre-prepared L-15 media could simply increase the osmolarity to harmful hypertonic levels, resulting in cell shrinkage, lysis, and overall lower cell viability. Additionally, since galactose induces higher levels of mitochondrial oxidative metabolism, cells could be more susceptible to reactive oxygen species which form from increased oxygen consumption (Galant et al., 2024).

Sources of Error:

One problem that could have influenced our results was noticeable precipitate formation in the culture flasks of both high galactose treatments. This is not conducive to strong culture growth since cells do not take up solid nutrients as efficiently as those in solution, meaning the galactose wouldn't have been efficiently taken up in these flasks. Precipitates can also remove other nutrients from solution by chelation. A suspected cause of precipitation was evaporation of media over time or temperature fluctuations (Millipore Sigma, 2025).

Another source of error in this experiment was inconsistent standard results for the BCA protein assay. Our calibration curve from prepared BSA standards had poor linearity, and one of the standards was an outlier and had to be disregarded (see Figure 4). Furthermore, our lysate samples fell in the low end of the linear range, which could mean poor accuracy or precision. Standard preparation could have been done with mis-calibrated micropipettes, but also the 60 ^{0}C

incubation step with the BCA working reagent could have been done for longer to ensure complete and consistent colour development for all standards and samples. It is also of note that after 14 days of growth, none of our three cultures reached confluent growth which was another source of inconsistency in this experiment.

Future Work:

For future research on this topic, the first improvement would be to complete each assay in triplicate over three days. Ideally, there would also be more and higher galactose treatments, perhaps up to 200 mM, and multiple culture flasks for each treatment. A scratch assay to determine the effect of galactose on cell migration could also provide more insight. Completing various assays including an MTT or an invasion assay could also be performed with different treatments to expand on the knowledge about protein content, survival, and metabolism. Within a larger scope, investigating the expression of specific mitochondrial metabolic proteins in response to high galactose concentration would be interesting, and much more relevant for comparison to previous work in the literature.

Conclusion:

This study investigated the effects of increased D-galactose concentrations on the viability and proliferation of endothelial cells. Cell viability decreased in a dose-dependent manner, with the highest galactose concentration (50 mmol/L) showing substantial cytotoxicity. The protein content peaked at the intermediate concentration (25 mmol/L), potentially indicating enhanced metabolic activity or stress-induced protein expression at moderate galactose levels. However, results should be interpreted with knowledge of experimental limitations which include galactose precipitation, inconsistencies in the BCA assay standard preparation, and small number of replicates. Overall, results suggest that moderate galactose concentrations stimulate protein production, and high galactose concentrations impair cell survival. Future studies are necessary to clarify the cellular mechanisms involved and validate these preliminary observations.

References:

Aguer, C., Gambarotta, D., Mailloux, R. J., Moffat, C., Dent, R., McPherson, R., Harper, M. E. (2011). Galactose Enhances Oxidative Metabolism and Reveals Mitochondrial Dysfunction in Human Primary Muscle Cells. *PLOS One.* https://doi.org/10.1371/journal.pone.0028536

Galant, L. S., Doblado, L., Radi, R., Fabro de Bem, A., Monsalve, M. (2024). Culture of Bovine Aortic Endothelial Cells in Galactose Media Enhances Mitochondrial Plasticity and Changes Redox Sensing, Altering Nrf2 and FOXO3 Levels. *Antioxidants*. (13) 873-887. https://doi.org/10.3390/antiox13070873

Gao, J., Liu, P., Shen, Z., Xu, K., Wu, C., Tian, F., Chen, M., Wang, L., & Li, P. (2021). Morroniside Promotes PGC-1α-Mediated Cholesterol Efflux in Sodium Palmitate or High Glucose-Induced Mouse Renal Tubular Epithelial Cells. *BioMed Research International*, *2021*, 1–14. https://doi.org/10.1155/2021/9942152

Lisec, B., Bozic, T., Santek, I., Markelc, B., Vrecl, M., Frangez, R., Cemazar, M. (2024). Characterization of two distinct immortalized endothelial cell lines, EA.hy926 and HMEC-1, for in vitro studies: exploring the impact of calcium electroporation, Ca^{2+} signaling and transcriptomic profiles. *Cell communication and signaling: CCS*, 22(1), 118. https://doi.org/10.1186/s12964-024-01503-2

Millipore Sigma [online]. (2025). Common Cell Culture Problems: Precipitates. [cited 2025 March 20] Available from: https://www.sigmaaldrich.com/CA/en/technical-documents/technical-article/cell-culture-and-cell-culture-analysis/mammalian-cell-culture/troubleshooting-precipitates

Nelson, D. L., Cox, M. M. (2017). Lehninger Principles of Biochemistry. W. H. Freeman and Company, New York.

Sigma Aldrich [online]. (2007). L-15 Medium Leibovitz. [cited 2025 March 20] Available from: https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/product/documents/232/017/l4386pis.pdf

Urban, J. (2025). Biology 3520: Cell Physiology Laboratory Manual Winter 2025. Thompson Rivers University, Kamloops, BC.

Yorek, M. A., Stefani, M. R., & Moore, S. A. (1991). Acute and chronic exposure of mouse cerebral microvessel endothelial cells to increased concentrations of glucose and galactose: Effect on myo-inositol metabolism, PGE2 synthesis, and transport activity. *Metabolism*, *40*(4), 347–358. https://doi.org/10.1016/0026-0495(91)90144-l

Zhang, L.-C., Wei, Z.-B., & Tang, S.-F. (2020). Knockdown of the Long Noncoding RNA LUCAT1 Inhibits High-Glucose-Induced Epithelial-Mesenchymal Transition through the

miR-199a-5p–ZEB1 Axis in Human Renal Tubular Epithelial Cells. *BioMed Research International*, *2020*, 1–8. https://doi.org/10.1155/2020/8895003