University of Glasgow | College of Medical, Veterinary & Life Sciences

Head of College Scholars List Scheme

Summer Studentship 2019

Report Form

This report should be completed by the student with his/her project supervisor. It should summarise the work undertaken during the project and, once completed, should be sent by email to: maureen.bain@glasgow.ac.uk **within four weeks of the end of the studentship**.

1. Student
   Surname: Monika Forename: Petrauskyte
   E-mail address: 2318171p@student.gla.ac.uk

2. Supervisor

Surname:		Forename:	
1.	Nicklin		Stuart
2.	McArthur		Lisa

   E-mail address: stuart.nicklin@glasgow.ac.uk

3. Research Project Report

   3.1 Project Title (maximum 20 words):

   Developing gene therapy to prevent post-myocardial infarction heart failure

   3.2 Project Lay Summary (copied from application):

   Following a heart attack loss of muscle cells occurs, a scar forms and the heart is unable to pump effectively. If untreated heart failure develops. Although treatments exist, they are not effective in everyone and heart failure rates are increasing, leading to a requirement to develop new therapies. We have identified a hormone circulating in blood which directly improves the heart's ability to pump. We use gene therapy with a special virus to deliver the hormone directly to the heart. Here we will test this gene therapy in cultured cardiomyocytes to confirm it works before testing in animal models.

   3.3 Start Date: 13/05/19 Finish Date: 22/06/19

3.4 Original project aims and objectives (100 words max):

We are developing novel next generation Ang-(1-9) expressing gene transfer vectors in which a mCherry reporter gene is co-expressed with Ang-(1-9) in order to tag cardiomyocytes facilitating study of gene transfer directly in transduced cells.

The aim of this proposed project is to characterize the novel gene transfer vectors in cardiomyocytes in vitro for effects on cell growth and viability.

We hypothesize that gene transfer of next generation Ang-(1-9)-reporter genes will facilitate direct identification of transduced cardiomyocytes via fluorescence microscopy and direct assessment of the effects on cell growth and viability leading to new knowledge about how the gene therapy protects the heart.

1.1 Methodology: Summarise and include reference to training received in research methods etc. (250 words max):

Generating Adenovirus Type 5 (Ad5) vector expressing Angiotensin-(1-9)

1. pShuttleCMVAng-(1-9) and pShuttleCMVFurin plasmids that express mCherry were mapped using *Kpn*I and *Hin*dIII. Gel electrophoresis was used to confirm correct configuration.

2. Transformation: plasmids were used to heat shock transform DH5α competent cells.

3. Plasmid preparation: minipreparation and maxipreparation were performed. Gel electrophoresis was used to confirm plasmids were present and correct. NanoDrop was used to measure DNA quality and concentration.

4. Cell culture: HeLa cells were grown in minimum essential medium enriched with fetal bovine serum, penicillin/streptomycin solution, sodium pyruvate and L-glutamine; hemocytometer was used to count cells.

5. Xfect™ Transfection (Clontech): experiments were carried out in triplicate using pShuttleCMVAng-(1-9) and pShuttleCMVFurin DNA from maxi preparations. Five µg of DNA was transfected per well. pGFP, Xfect only and media were used as controls. Optimization of transfection included repeating the experiment in triplicates in 12-well plates using 1.75 uL and 2.5 uL of DNA. AAVAng-(1-9) and AAVFurin plasmids expressing mCherry were added as control. TaqMan RT-qPCR was performed after RNA isolation with QIAzol to confirm expression of transgenes.

6. Nikon Eclipse TS100 was used for fluorescence imaging.

First steps of generating recombinant adenoviruses

1. pShuttleCMVAng-(1-9) and pShuttleCMVFurin DNA from the maxi preparations was digested with *Pme*I and analysed by gel electrophoresis. The correct size DNA fragments were cut out and Wizard® SV Gel and PCR Clean-Up System (Promega) was used to purify the DNA.

2. Transformation: the purified DNA was used to transform (electroporation) BJ5183-AD-1 competent cells. The procedure was repeated using phenol-chloroform purified DNA.

3. Plasmid minipreparation was performed and restriction digest and gel electrophoresis used to identify positive and negative clones.

3.5 Results: Summarise key findings (300 words max). Please include any relevant tables or images as an appendix to this report:

Generating Adenovirus Type 5 (Ad5) vector expressing Angiotensin-(1-9)

Gel electrophoresis of pShuttleCMVAng-(1-9) and pShuttleCMVFurin shows that plasmid configurations were correct: 841 bp band in figure 1 and 883 bp band in figure 2 represent Furin and Ang-(1-9) respectively (for figures, see Appendices).

Bacterial growth on agar plates with kanamycin indicated that transformation of DH5α with pShuttle plasmid DNA was successful. 841 bp and 883 bp bands were still present in gel images after both minipreparation (figures 3 and 4) and maxipreparation (figures 5 and 6) confirming that all chosen colonies of DH5α cells contain the pShuttle plasmids.

Following maxipreparation, pShuttleCMVAng-(1-9) and pShuttleCMVFurin clones with highest DNA concentration were transfected into HeLa cells and images were captured using a Nikon Eclipse TS100 fluorescent microscope. In 6-well plates, fluorescence was observed in both pShuttle and pGFP wells for both Furin and Ang-(1-9) showing that transfection was successful (figure 7). However, the expression of mCherry (red) was lower compared to that of GFP (green) so it was decided to further optimise transfection. After optimisation, although AAVAng-(1-9), AAVFurin and pGFP controls were detectable, no fluorescence could be detected in pShuttleCMVAng-(1-9) and pShuttleCMVFurin (figure 8). Next, TaqMan RT-qPCR was performed and detected and quantified mCherry expression from pShuttle vectors (figure 9), confirming that the plasmids were correct and could be taken forward to produce adenoviral vectors.

First steps of generating recombinant adenoviruses

Bacterial growth on agar plates with kanamycin indicated that transformation of BJ5183-AD-1 with phenol-chloroform purified pShuttle plasmid DNA was successful. Gel images after minipreparation and restriction digest (figures 10, 11 and 12) showed that 5 colonies were

positive for pShuttleCMVFurin and 2 colonies were positive for pShuttleCMVAng-(1-9). Positive clones were then successfully retransformed into DH5α competent cells for efficient large scale production of plasmid DNA for transfection into 293 cells to produce adenoviral vectors.

3.6 Discussion (500 words max):

Myocardial infarction (MI) occurs when the flow of blood to the heart is blocked leading to a permanent damage to the heart muscle. Following MI, cardiac remodelling takes place initially enabling the heart to compensate for damage, however, in most cases it leads to heart failure (HI) and effective therapies which stabilise remodelling are necessary (Azevedo et al., 2016).

Angiotensin-(1-9) is a peptide member of counter-regulatory renin angiotensin system (RAS) axis. It is known to mediate anti-hypertrophic effects in cardiomyocytes and to reduce cardiac fibrosis which makes it a novel therapeutic target in cardiovascular diseases (McKinney et al., 2014).

This project focused on preparation for generating an Adenovirus Type 5 (Ad5) vector in which Angiotensin-(1-9) is co-expressed with a mCherry reporter gene. This novel approach allows to tag cardiomyocytes this way facilitating study of gene transfer directly in transduced cells. Since the preparation went smoothly, there was enough time to go through the first steps of generating a recombinant adenoviral genome making successfully re-transformed DH5α competent cells the final and the most important result of this project as this experiment successfully produced intact adenoviral genome DNA which could be transfected into 293 cells to produce the final adenoviral vector for the future research of the group.

Although preparation went smoothly, some experiments did not work or the outcome was not as expected. Despite all transfections being successful (as indicated by fluorescence in GFP control wells and detection of mCherry from pShuttleCMVAng-(1-9) and pShuttleCMVFurin) in the initial experiments, fluorescence in pShuttleCMVFurin and pShuttleCMVAng-(1-9) wells was absent (12 well plates) compared to controls when optimisation experiments were performed. We speculate the latter might be due to different sizes of plasmids (pShuttle 8426 bp for Ang-(1-9), pGFP 3344 bp) and different emission peaks (GFP 509 nm, mCherry 610 nm) which may have impact on sensitivity of Nikon Eclipse TS100 used for imaging since it is optimal for detection of GFP, but can only weakly detect mCherry. However, using a more sensitive technique of RT- qPCR indicated that mCherry was expressed in both pShuttleCMVAng-(1-9) and pShuttleCMVFurin confirming the plasmids were correct. Furthermore, some transformations of BJ5183-AD-1 competent cells did not work. We assume this might be the case of unsuccessful electroporation and/or DNA still being contaminated after a clean-up prior to ligation. This issue was overcome by repeating the experiment using DNA cleaned using phenol/chloroform extraction.

In conclusion, the project was successful and all goals set were achieved. The research will be continued by the group. The following steps will be transfection of recombinant pAdEasy-

1 and pShuttle Furin/pShuttle Ang-(1-9) adenoviral genomes generated during this project into low passage 293 cells for derivation of adenovirus stocks and generation of crude adenovirus stocks.

4. Reflection by the student on the experience and value of the studentship (300 words max):

The experience I gained during the six weeks of internship is incredibly valuable and went above and beyond my expectations. All members of BHF GCRC were very kind and helpful, making my first serious steps in lab environment much easier and less scary.

I learned a lot of new techniques (e.g., cell culture, gel electrophoresis, bacterial transformation, plasmid preparation, transfection, etc.) that are widely used and will be essential for my undergraduate years, future studies and career. I was taught that in science there is no such thing as failure and I am very grateful I was allowed to make mistakes and try to figure out what went wrong on my own. Now I feel much more confident and comfortable working without constant supervision and have some experience in experiment planning which is one of the most important skills for any researcher.

However, this internship was more than working in a lab. I was surrounded by enthusiastic and ambitious people who inspired me to keep working hard and encouraged me to try make the most of any opportunity (such as offered integrated master's degree). I was introduced into many research areas that are fascinating completely new to me and I was shown how important networking is.

Overall, these six weeks were very pleasant and enjoyable. I feel like I learned more than during the first two years of university and I am looking forward to coming back to working in a lab soon.

5. Dissemination: (note any presentations/publications submitted/planned from the work):

6. Signatures:

Supervisor Date Student Date

03/07/2019

03/07/19

References:

Azevedo, P., Polegato, B., Minicucci, M., Paiva, S. and Zornoff, L. (2016). Cardiac Remodeling: Concepts, Clinical Impact, Pathophysiological Mechanisms and Pharmacologic Treatment. Arquivos Brasileiros de Cardiologia.

McKinney, C., Fattah, C., Loughrey, C., Milligan, G. and Nicklin, S. (2014). Angiotensin-(1–7) and angiotensin-(1–9): function in cardiac and vascular remodelling. Clinical Science, 126(12), pp.815-827.

Appendix A

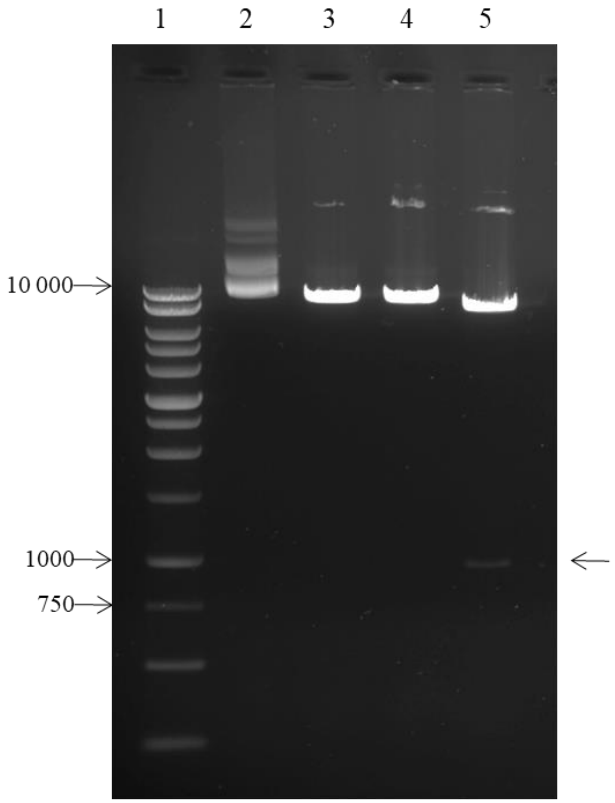

Figure 1. Agarose gel electrophoresis (0.8%) of *Kpn*I and *Hin*dIII digested pShuttleCMVFurin. Lane 1 – 1 kb DNA ladder, lane 2 – uncut pShuttleCMVFurin DNA, lane 3 – *Kpn*I, lane 4 – *Hin*dIII, lane 5 – *Kpn*I and *Hin*dIII. Arrow on the right indicates Furin (841 bp) is present.

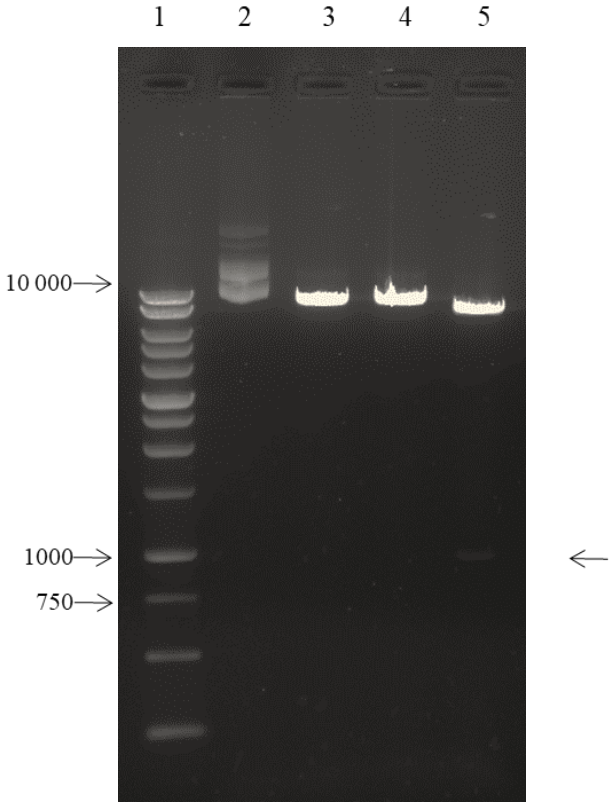

Figure 2. Agarose gel electrophoresis (0.8%) of *Kpn*I and *Hin*dIII digested pShuttleCMVAng-(1-9). Lane 1 – 1 kb DNA ladder, lane 2 – uncut pShuttleCMVAng-(1-9) DNA, lane 3 – *Kpn*I, lane 4 – *Hin*dIII, lane 5 – *Kpn*I and *Hin*dIII. Arrow on the right indicates Ang-(1-9) (883 bp) is present.

Appendix B

Sample ID	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
Furin clone 1	163.91	3.278	1.741	1.88	2.13	50.00	230	1.537	0.030
Furin clone 2	204.64	4.093	2.222	1.84	1.70	50.00	230	2.407	0.516
Furin clone 3	193.12	3.862	2.070	1.87	2.01	50.00	230	1.918	0.147
Furin clone 4	147.39	2.948	1.566	1.88	2.06	50.00	230	1.433	0.109
Ang-(1-9) clone 1	207.92	4.158	2.222	1.87	2.06	50.00	230	2.022	0.231
Ang-(1-9) clone 2	233.63	4.673	2.466	1.90	2.21	50.00	230	2.111	0.086
Ang-(1-9) clone 3	245.11	4.902	2.550	1.92	2.17	50.00	230	2.260	0.076
Ang-(1-9) clone 4	229.36	4.587	2.453	1.87	1.88	50.00	230	2.441	0.500

Table 1: NanoDrop data of DNA concentrations after minipreparation (PureYield).

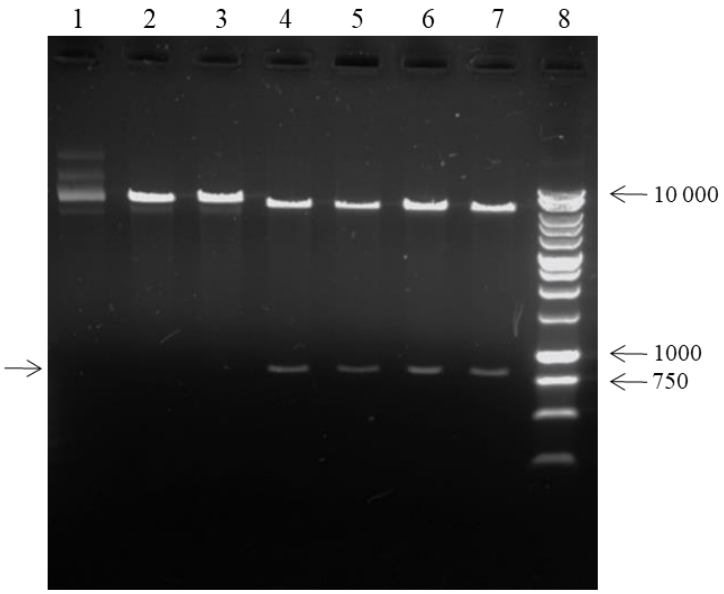

Figure 3. Agarose gel electrophoresis (0.8%) of *Kpn*I and *Hin*dIII digested pShuttleCMVFurin after minipreparation. Lane 1 – uncut pShuttleCMVFurin DNA, lane 2 – clone 2 *Kpn*I, lane 3 – clone 2 *Hin*dIII, lane 4 – clone 2 *Kpn*I and *Hin*dIII, lane 5 – clone 1 *Kpn*I and *Hin*dIII, lane 6 – clone 3 *Kpn*I and *Hin*dIII, lane 7 – clone 4 *Kpn*I and *Hin*dIII, lane 8 – 1 kb DNA ladder. Arrow on the left indicates Furin (841 bp) is present.

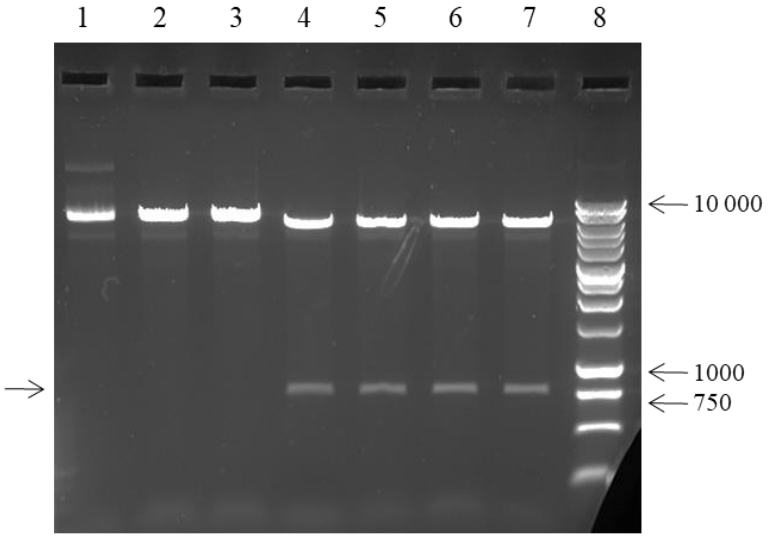

Figure 4. Agarose gel electrophoresis (0.8%) of *Kpn*I and *Hin*dIII digested pShuttleCMVAng-(1-9) after maxipreparation. Lane 1 – uncut pShuttleCMVANg-(1-9) DNA of clone 2, lane 2 – clone 2 *Kpn*I, lane 3 – clone 2 *Hin*dIII, lane 4 – clone 2 *Kpn*I and *Hin*dIII, lane 5 - clone 1 *Kpn*I and *Hin*dIII, lane 6 – clone 3 *Kpn*I and *Hin*dIII, lane 7 – clone 4 *Kpn*I and *Hin*dIII, lane 8 - 1 kb DNA ladder. Arrow on the left indicates Ang-(1-9) (883 bp) is present.

Appendix C

Sample ID	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
Ang-(1-9) clone 2	3916.13	78.323	41.731	1.88	2.37	50.00	230	33.066	0.317
Ang-(1-9) clone 3	4707.98	94.160	51.780	1.82	2.28	50.00	230	41.300	0.415
furin 2	4315.84	86.317	45.396	1.90	2.44	50.00	230	35.389	0.387
furin 3	2785.54	55.711	28.845	1.93	2.43	50.00	230	22.908	0.339

Table 2: After minipreparation, clones with highest concentration and least contamination were chosen for maxipreparation. Table represents NanoDrop data of DNA concentrations after maxipreparation (PureYield).

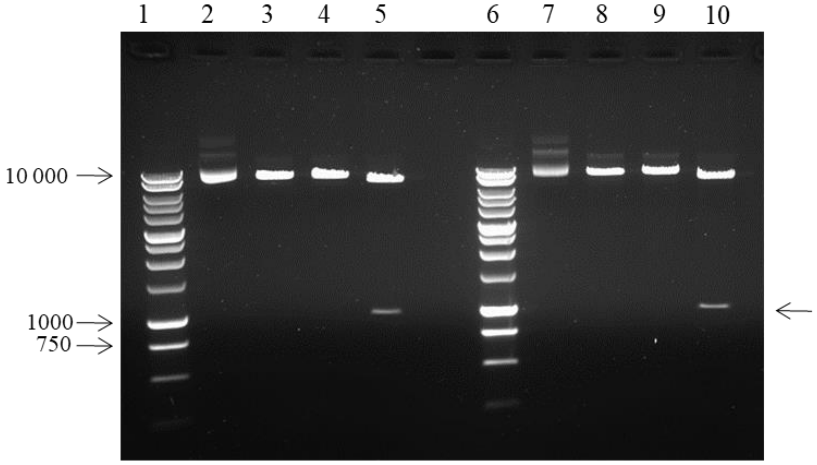

Figure 5. Agarose gel electrophoresis (0.8%) of *Kpn*I and *Hin*dIII digested pShuttleCMVFurin after maxipreparation. Lane 1 – 1 kb DNA ladder, lane 2 - uncut pShuttleCMVFurin DNA of clone 2, lane 3 – clone 2 *Kpn*I, lane 4 – clone 2 *Hin*dIII, lane 5 – clone 2 *Kpn*I and *Hin*dIII, lane 6 - 1 kb DNA ladder, lane 7 - uncut pShuttleCMVFurin DNA of clone 3, lane 8 – clone 3 *Kpn*I, lane 9 – clone 3 *Hin*dIII, lane 10 – clone 3 *Kpn*I and *Hin*dIII. Arrow on the right indicates Furin (841 bp) is present.

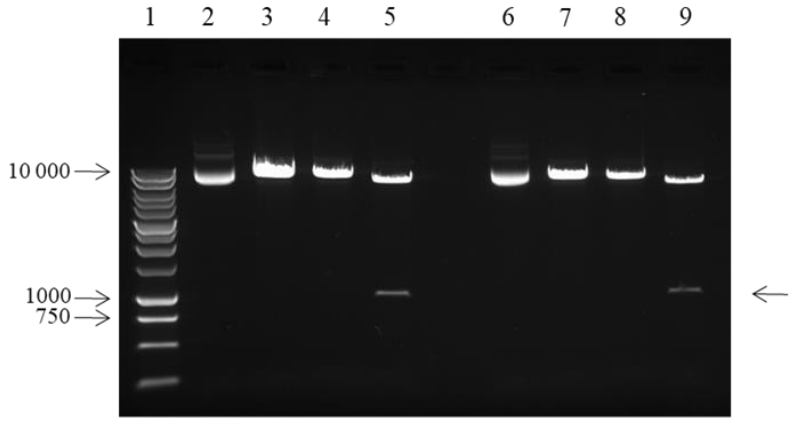

Figure 6. Agarose gel electrophoresis (0.8%) of *Kpn*I and *Hin*dIII digested pShuttleCMVAng-(1-9) after maxipreparation. Lane 1 – 1 kb DNA ladder, lane 2 - uncut pShuttleCMVAng-(1-9) DNA of clone 2, lane 3 – clone 2 *Kpn*I, lane 4 – clone 2 *Hin*dIII, lane 5 – clone 2 *Kpn*I and *Hin*dIII, lane 6 - uncut pShuttleCMVAng-(1-9) DNA of clone 3, lane 7 – clone 3 *Kpn*I, lane 8 – clone 3 *Hin*dIII, lane 9 – clone 3 *Kpn*I and *Hin*dIII. Arrow on the right indicates Ang-(1-9) (883 bp) is present.

Appendix D

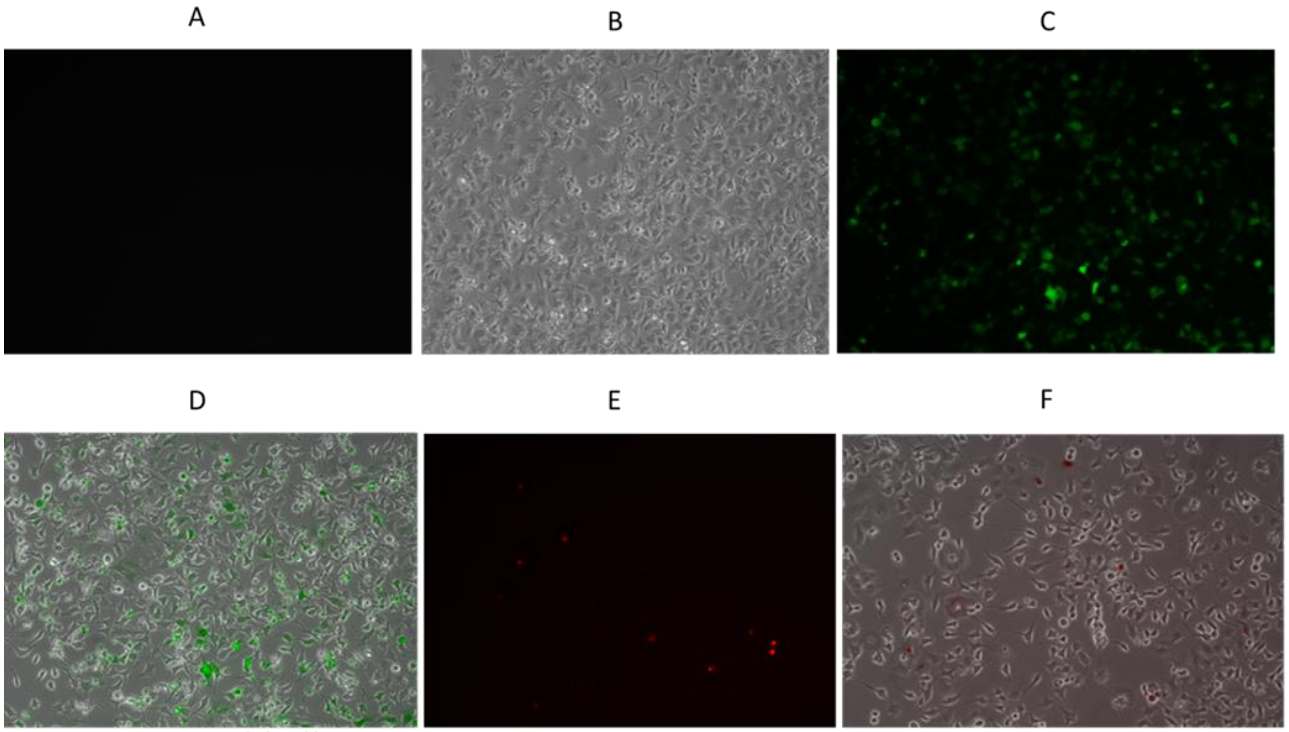

Figure 7. Xfect™ Transfection (Clontech) into HeLa cells. A – no DNA control, B – no DNA control, composition of bright field and fluorescent images, C – 5 µl GFP control plasmids; fluorescent light, exposure 1, D - 5 µl GFP control plasmids; composition of bright field and fluorescent images, E - 5 µl pShuttleCMVFurin; fluorescent light, exposure 3, F - 5 µl pShuttleCMVFurin; composition of bright field and fluorescent images.

Appendix E

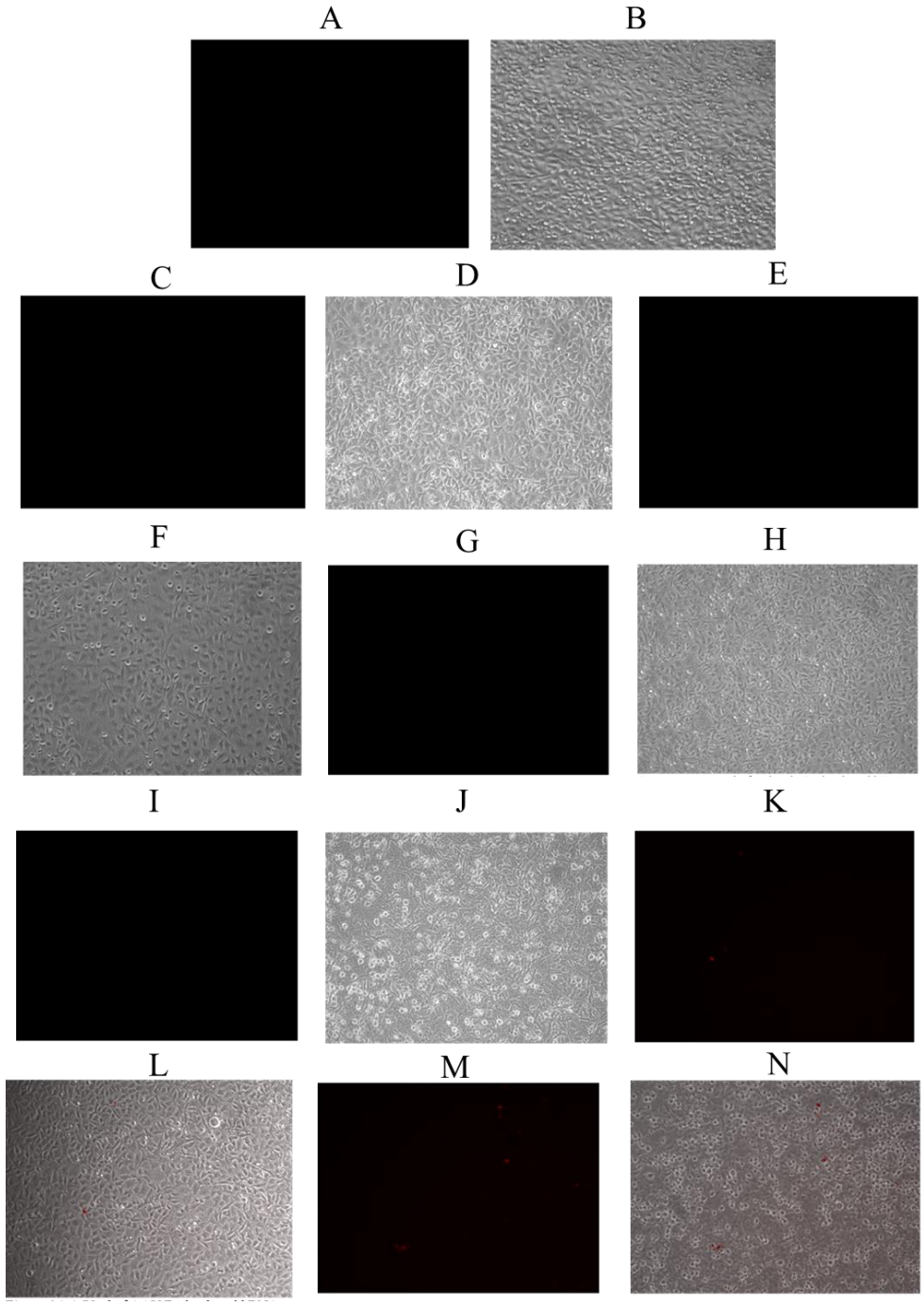

Figure 8. Xfect™ Transfection (Clontech) into HeLa cells. A – no DNA control for 1.75 µl of pShuttleCMVFurin; fluorescent light, B – no DNA control for 1.75 µl of pShuttleCMVFurin; composition of bright field and fluorescent images, C - no DNA control for 2.5 µl of pShuttleCMVFurin; fluorescent light, D - no DNA control for 2.5 µl of pShuttleCMVFurin; composition of bright field and fluorescent images, E – medium only; fluorescent light, F – medium only, composition of bright field and fluorescent images, G – 1.75 µl pShuttleCMVFurin; fluorescent light, H - 1.75 µl pShuttleCMVFurin; composition of bright field and fluorescent images, I – 2.5 µl pShuttleCMVFurin; fluorescent light, J – 2.5 µl pShuttleCMVFurin; composition of bright field and fluorescent images, K - 1.75 µl AAV Furin; fluorescent light, L – 1.75 µl AAV Furin; composition of bright field and fluorescent images, M – 2.5 µl AAV Furin; fluorescent light, N – 2.5 µl AAV Furin; composition of bright field and fluorescent images.

Appendix F

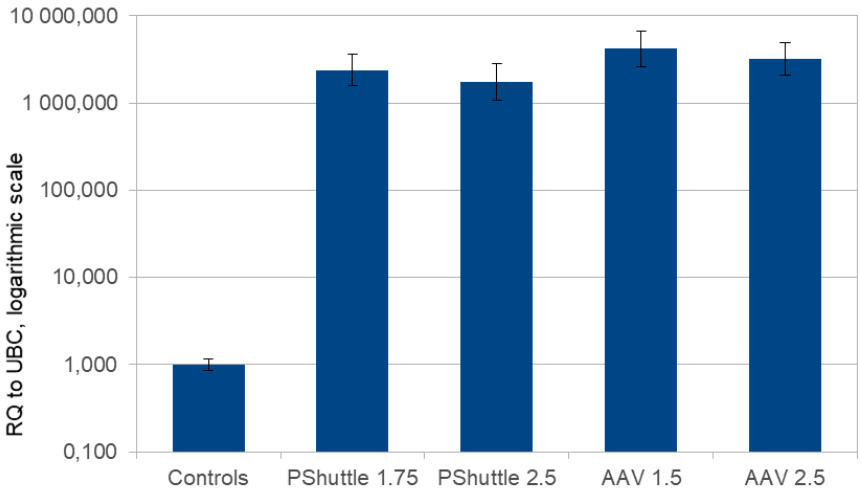

Figure 9. Relative expression of pShuttleCMVFurin and AAV Furin plasmids. RQ max – positive error values, RQ min – negative error values.

Appendix G

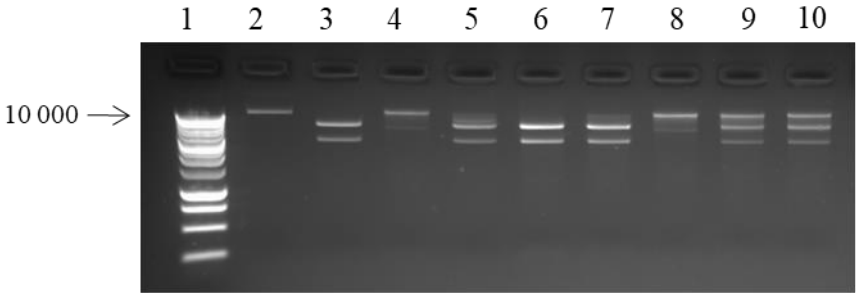

Figure 10. Agarose gel electrophoresis (0.8%) of *Pme*I digested pShuttleCMVAng-(1-9) after re-transformation and minipreparation. Lane 1 – 1 kb DNA ladder, lane 2 – uncut pShuttleCMVAng-(1-9), lane 3 – *Pme*I cut pShuttleCMVAng-(1-9), lanes 4 and 8 – positive clones, lanes 3, 5, 6, 7, 9, and 10 – negative clones.

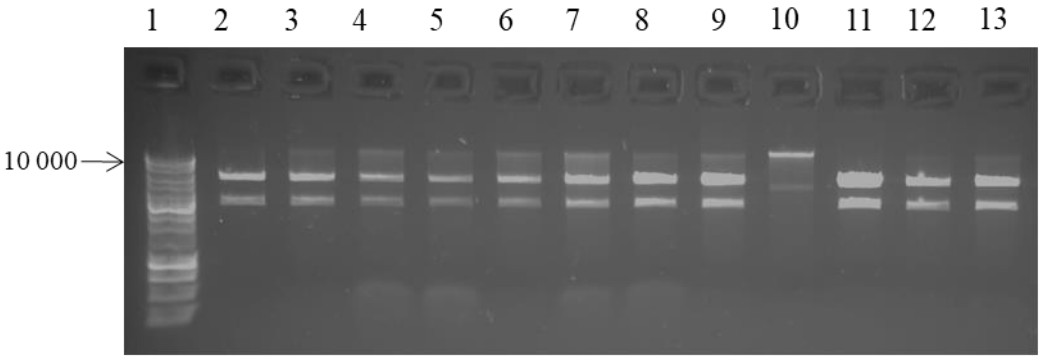

Figure 11. Agarose gel electrophoresis (0.8%) of *Pme*I digested pShuttleCMVFurin plasmids after re-transformation and minipreparation. Lane 10 is the only positive clone.

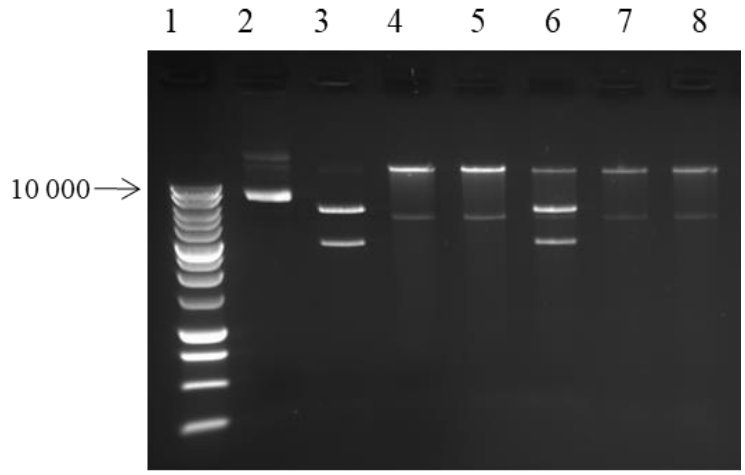

Figure 12. Agarose gel electrophoresis (0.8%) of PmeI digested pShuttleCMVFurin after re-transformation and minipreparation. Lane 1 – 1 kb DNA ladder, lane 2 – uncut pShuttleCMVFurin, lane 3 – PmeI cut pShuttleCMVFurin, lanes 4, 5, 7 and 8 – positive clones, lane 3 – negative clone.