The Development of a Green Fluorescent Protein Reporter System for Apoptosis Repressor with Caspase Recruitment Domain Promoter Analysis

by

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Statement of Contributions

Dr. Joe Quadrilatero assisted in the editing of this document.

Abstract

Apoptosis is a form of controlled cell death that is important to tissue homeostasis. There are a variety of pro- and anti-apoptotic proteins that play an important role in the regulation of cell death. Apoptosis repressor with caspase recruitment domain (ARC) is a potent anti-apoptotic protein that has the ability to inhibit cell death through all the major apoptotic pathways. ARC is highly expressed in post-mitotic tissue such as cardiac muscle, neurons, and skeletal muscle, but is expressed at much lower levels in other tissues. However, ARC protein levels can increase dramatically when some tissues become cancerous. Research has shown that this increase in ARC protein content results in resistance to chemotherapeutic drugs. Additionally, decreasing ARC protein in cancer cell lines has been shown to be effective in increasing the sensitivity of these cancer cells to apoptosis-inducing agents. Therefore, ARC presents an attractive therapeutic target. The development of a reporter system that is driven by the ARC promoter region would allow for the monitoring of ARC expression during a high-throughput drug screen. Green fluorescent protein (GFP) is a valuable tool commonly used to analyse gene expression in cells and presents numerous advantages over a luciferase-based reporter system. First, GFP can be detected without the lysing the cells and without the addition of costly substrates. Second, fluorescence can be detected by a simple plate reader assay. Thus, a GFP reporter plasmid under the control of the ARC promoter region was developed. However, GFP production was limited in cells transfected with the reporter plasmid and significant changes in GFP fluorescence as ARC protein levels increased was not detected by the fluorescent plate reader assay. Ultimately, more research is needed to develop a reporter plasmid that responds to ARC promoter activation and can be detected by a plate reader assay.

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Introduction

1 Apoptosis

Apoptosis is a form of controlled cell death that is required for the maintenance of tissue homeostasis (1). It is a highly conserved process critical in the formation of tissue during development and throughout tissue remodelling (2). Additionally, apoptosis plays an important role in ensuring that damaged or mutated cells are eliminated and therefore prevented from causing further harm (2). When a cell is highly stressed, such as when DNA damage occurs or following growth factor depletion, apoptotic signalling pathways may be activated (3). In general, these pathways ultimately converge onto pro-apoptotic proteolytic enzymes known as caspases, resulting in the degradation of cellular proteins and ultimately cell death without a large inflammatory immune response (3,4). The different apoptotic signalling pathways involve distinct components of the cell (5).

1.1 The intrinsic pathway

Mitochondria play an integral role in the intrinsic apoptotic signalling pathway, which involves the release of pro-apoptotic proteins (6). The release of these proteins occurs as a result of mitochondrial outer membrane permeabilization (MOMP) (7).

The B-cell lymphoma 2 (BCL-2) family of proteins play an important role in the execution of cell death through the mitochondria (8). BCL-2 proteins are divided into 3 categories: pro-apoptotic, anti-apoptotic, and BCL-2 homology 3-only (BH3-only) (9). The pro-apoptotic proteins BCL-2 associated x protein (BAX) and BCL-2 antagonist killer (BAK) are directly responsible for the execution of cell death through the creation of a BAX/BAK pore in the outer mitochondrial membrane (OMM), which allows for the release of mitochondrial proteins (10). Anti-apoptotic proteins, such as B-cell lymphoma-extra large (BCL-XL) and BCL-2, bind to

pro-apoptotic factors to prevent them from initiating cell death under normal conditions (11). While the exact roles of BH3-only proteins are still under investigation, it is clear that they act as critical regulators in the intrinsic apoptotic response by directly activating the pro-apoptotic proteins BAX and BAK or by binding to anti-apoptotic proteins to inhibit their ability to prevent apoptosis (12–15).

When an apoptotic signalling pathway is activated, BH3-only proteins are produced and directed to execute their particular role, whether that is to displace the anti-apoptotic proteins bound to BAX and BAK, bind to free anti-apoptotic proteins to neutralize their effect, or directly activate BAX and BAK (16). BAX is a cytosolic protein under normal conditions, but once BAX is activated, it translocates to the mitochondria, inserts into the OMM, oligomerizes with other BAX molecules, and creates a pore (17). Similarly, BAK, which resides on the mitochondrial membrane, oligomerizes with other BAK molecules when it is activated to create a pore in the OMM (17).

Mitochondrial fission and its associated protein Drp-1 have also been shown to be involved in apoptosis (18). While there appears to be a relationship between mitochondrial fission and MOMP, the details remain unclear (18). However, there is evidence to suggest that Drp-1 translocates from the cytosol to the mitochondrial membrane with the help of p53 upregulated modulator of apoptosis (PUMA) when an apoptotic stimulus is received (19). This results in the fragmentation of mitochondria and may lead to the release of pro-apoptotic proteins (18,19).

The pore created in the OMM allows for the release of pro-apoptotic proteins such as cytochrome *c*, apoptosis-inducing factor (AIF), endonuclease G (Endo G), and second mitochondria-derived activator of caspases (SMAC) (20). These proteins play an important role in cell function while located in the mitochondria, but each protein plays a different role in the

execution of cell death when they are released into the cytosol (21). Cytochrome *c* combines with apoptotic protease activating factor 1 (Apaf-1) and the initiator caspase, caspase-9, with the help of dATP to create the apoptosome (22). The apoptosome activates the executioner caspase caspase-3, which leads to protein degradation and cell death (23). Additionally, the release of cytochrome *c* causes dysfunction of the electron transport chain, leading to a decrease in ATP and an increase in free radical production, which also contributes to the death of the cell (24,25). When released from the mitochondria, AIF and Endo G translocate to the nucleus and cause DNA fragmentation, an important component of the cell death process (26,27). AIF and Endo G can also be released from the mitochondria independent of caspase activation to induce cell death (26,27). Furthermore, SMAC acts as an antagonist of X-linked inhibitor of apoptosis protein (XIAP) and other inhibitor of apoptosis proteins (IAPs) to block their anti-apoptotic function (28). Overall, these mitochondrial proteins play a critical role in the execution of cell death through the intrinsic pathway.

1.2 The extrinsic pathway

The extrinsic apoptotic signalling pathway is reliant on the binding of ligands, including Fas, tumour necrosis factor (TNF) and TNF-related apoptosis-inducing ligand (TRAIL), to cell surface death receptors (29). The binding of the specific ligand to the appropriate receptor results in death receptor activation and recruitment of the associated adaptor protein, including Fas associated protein with death domain (FADD) and tumour necrosis factor receptor type 1-associated death domain (TRADD) (29). TRADD/FADD attach to the death receptor through the binding of the death domains (DD), which are located on the cytosolic portion of the death receptor (29,30). Procaspase-8, an initiator caspase, binds to FADD through the death

effector domains (DED) located on both proteins (29,30). This forms the death inducing signalling complex (DISC) and leads to the activation of caspase-8 (Figure 1) (30).

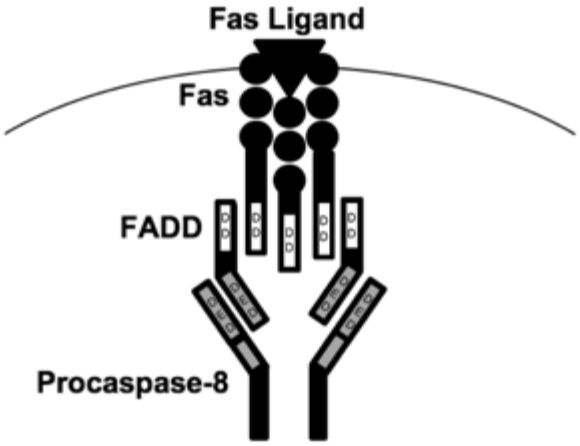


Figure 1: The death inducing signalling complex. Adapted from Nam et al., 2004 (30).

Activated caspase-8 can cleave the BH3-only protein BID to form truncated BID (tBID), which translocates to the mitochondria, activates the intrinsic pathway, and ultimately amplifies the apoptotic signal received through the death receptor (31). Independent of the intrinsic pathway, caspase-8 can directly activate caspase-3, which will lead to cell degradation (32).

1.3 The endoplasmic reticulum stress pathway

The accumulation of unfolded proteins in the endoplasmic reticulum (ER) can create a stressful cellular environment and cause calcium to be released into the cytosol (33). If high cytosolic calcium levels are maintained, cell death can be initiated by activation of the cysteine protease m-calpain (34). Furthermore, caspase-12 may be activated directly by ER stress through the dissociation from its adaptor protein at the ER membrane or activated by m-calpain (35,36). Activated caspase-12 can activate both caspase-9 and caspase-3, which will lead to cell death (37–39). Additionally, the expression of the transcription factor C/EBP homologous protein (CHOP) is induced during times of ER stress (40). CHOP may act to as a transcription

factor to alter the expression of anti- or pro-apoptotic genes, disrupt the cellular redox state by affecting glutathione production, or play a role in protein-protein interactions to cease cell proliferation (41).

In addition to its other functions, mitochondria play an important role in cellular calcium regulation in some cells (42). Mitochondria have the ability to sequester cytosolic calcium and therefore aid in controlling cytosolic calcium levels (43). However, if the amount of calcium sequestered exceeds the amount that the mitochondria can tolerate, the permeability transition pore (PTP) may open (43,44). The PTP is created by the voltage dependent anion channel (VDAC) on the outer mitochondrial membrane and adenine nucleotide translocase (ANT) on the inner mitochondrial membrane, which associates with cyclophilin-D (CyP-D) (45). The assembly and opening of this complex can cause a disruption of the mitochondrial membrane potential, the release of pro-apoptotic proteins into the cytosol, and subsequent cell death through the intrinsic pathway (45).

1.4 The DNA damage pathway

DNA damage can result in cell death through the activation of the tumour suppressor p53 (46). Activated p53 acts as a transcription factor in the nucleus and promotes the transcription of pro-apoptotic and BH3-only proteins such as BAX and PUMA, which can ultimately lead to cell death through the intrinsic apoptotic pathway (47,48). Additionally, p53 can lead to the production of p53-induced protein with a death domain (PIDD), which can interact with receptor-interacting protein (RIP)-associated ICH-1/CED-3 homologous protein with a death domain (RAIDD) and caspase-2 (49,50). The interaction of these proteins results in the production of the PIDDosome, which activates caspase-2 (51,52). Activated caspase-2 can cleave the BH3-only protein BID to form tBID, which can activate the intrinsic apoptotic pathway

(50,53). Independent of its transcription factor role, cytosolic p53 has been shown to directly activate BAX and BAK, while inhibiting BCL-2 and BCL-XL (54).

2 Apoptosis Repressor with Caspase Recruitment Domain

Apoptosis repressor with caspase recruitment domain (ARC) is a unique and potent anti-apoptotic protein (55,56). The human ARC (NOL3) gene is located on chromosome 16 and produces a 208 amino acid protein with a molecular mass of 22.6 kD (56). While most other anti-apoptotic proteins appear to have a particular role in a specific apoptotic pathway, it has been shown that ARC has the ability to inhibit cell death through all the major apoptotic pathways (56).

2.1 Function

ARC consists of two domains that are integral to its function: a caspase recruitment domain (CARD) at the n-terminus, which allows ARC to bind to the CARD and death domains on other proteins, and a proline/glutamate (PE) rich region at the c-terminus, which binds cytosolic calcium and p53 (56,57). These two domains allow ARC to act as an inhibitor of cell death by impeding the action of the factors involved in the apoptotic signalling pathways (56).

The action of proteins vital to the intrinsic cell death pathway, including BAX, PUMA and BCL-2 associated death promoter (BAD), have been shown to be inhibited by ARC via the interaction of its CARD with the BH3 domains of PUMA and BAD and the c-terminus region of BAX (30,58). This inhibition prevents mitochondrial pore formation and the release of pro-apoptotic proteins. Further, PUMA plays an important role in the mitochondrial targeting of Drp-1 (19). With ARC bound to PUMA, Drp-1 is unable to translocate to the mitochondrial membrane and therefore is incapable of causing mitochondrial fission (19).

Furthermore, the ARC CARD domain can bind to the death domains located on the proteins involved in the DISC (30). This interaction can prevent DISC assembly, the activation of caspase-8, and therefore cell death through the extrinsic pathway (30). ARC can also bind directly to procaspase-8 through its death domain and inhibit its ability to be activated (55).

The ability of ARC to bind calcium in the PE region can decrease the levels of cytosolic calcium during times of ER cell stress (59). Therefore, these calcium levels may not reach the required stress threshold to initiate the ER stress response (59). Additionally, ARC has been shown to have the ability to suppress CHOP production in response to ER stress in pancreatic beta cells. However, the mechanism behind this suppression is not clear (60).

The DNA damage pathway can also be moderated by ARC through its ability to bind to the tetramerization domain of p53 via its PE region (61). This binding prevents p53 tetramerization and exposes a nuclear export signal, essentially inactivating p53 and exporting it from the nucleus, which eliminates its ability to act as a transcription factor for pro-apoptotic proteins (61). ARC additionally has the ability to bind to the death domain of caspase-2 through its CARD (55). Therefore, caspase-2 is unable to interact with PIDD and RAIDD to create the PIDDosome, ultimately preventing caspase-2 from being activated.

Together, the ability of ARC to inhibit apoptosis through multiple pathways makes it a potent regulator of cell death.

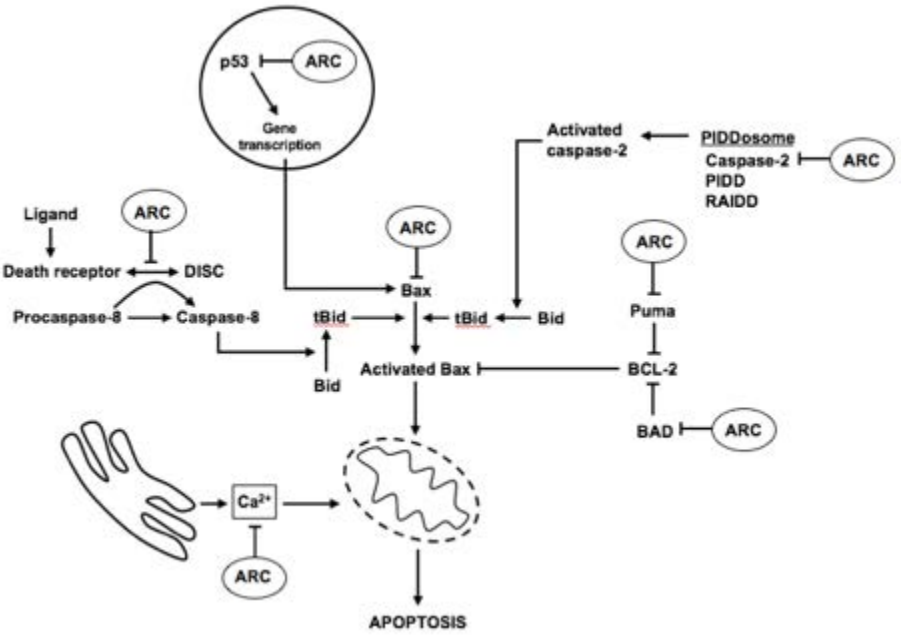


Figure 2: ARC has the ability to inhibit apoptosis through all the major cell death pathways. Adapted from Ludwig-Galezowska et al., 2011 (56).

2.2 Tissue expression

Another unique aspect of ARC is its distinct tissue expression (56). ARC is highly expressed in terminally differentiated tissue such as cardiac muscle, neurons, and skeletal muscle, but is expressed at lower levels in most other tissues (55,62). In tissues where expression levels are high, its anti-apoptotic function plays an important role (63–65). In the heart, ARC has been shown to play a cardioprotective role (66). In patients with heart failure, ARC expression is decreased in cardiac tissue, making cardiomyocytes more susceptible to apoptosis (67). Furthermore, there is evidence to show that overexpression of ARC in rat cardiomyocytes reduced the extent of damage and prevented apoptosis after cardiac injury (68). Interestingly, our lab has shown that ARC expression is low in mouse C2C12 myocytes but increases significantly during differentiation. With this increased expression, these differentiated myocytes are highly resistant to apoptosis-inducing agents (69). Overall, ARC clearly plays an important role in preventing apoptosis in tissues where cell death can be detrimental to its function. Conversely, high levels of

ARC expression in cancerous tissue has been associated with chemoresistance and poor patient prognosis (19,70–72).

2.3 Cellular localization

ARC has been found to be expressed in the cytosol, nucleus, and on the mitochondrial membrane (62,70,72,73). Interestingly, the cellular localization of ARC appears to be highly variable depending on the tissue and malignancy status (62,72,73). In normal rat cardiomyocytes, rat hippocampal neurons, and human skeletal muscle, ARC is primarily located in the cytosol, whereas the localization appears to be more variable when tissue becomes malignant (62,72,73). In some cancer cell lines, including HT116 human colon cancer cells, DU145 human prostate cancer cells, and HeLa human cervical cancer cells, ARC has been found predominantly in the nucleus (62). In MCF7 human breast cancer cells and OCI-AML3 human acute myeloid leukemia (AML) cells, ARC was shown to be equally present in both cytoplasmic and nuclear compartments (72,74). Similarly, in human colon cancer patient samples, ARC was found to be present in both the cytosol and nucleus (73). However, there appear to be differences in ARC localization depending on the cancer grade (73). In moderately differentiated human colon cancer samples, ARC was found to be highly concentrated in the nucleus, in contrast to poorly and well differentiated samples where it was concentrated in the cytosol (73). It is clear from these findings that the primary location of ARC in a cell varies depending on the type of tissue.

Furthermore, the cellular localization of ARC can change with phosphorylation (62,70). Casein kinase II (CK2) phosphorylates ARC at the T149 site and subsequently directs ARC to the mitochondrial membrane where is has been shown to bind to and inhibit caspase-8 (70). Whether phosphorylation of other amino acids residues on ARC are required for translocation to other cellular locations is yet to be determined (62).

2.4 Transcriptional regulation

There are a variety of factors that impact the production and function of ARC. It is important to note that this research has been done in a variety of cell lines from different animals, making it difficult to make definitive conclusions about ARC regulation since the ARC gene sequence and transcription factors vary between rats, mice, and humans.

At the transcriptional level, p53 has the ability to inhibit ARC transcription by binding to the promoter region on the rat ARC (NOL3) gene (58). Conversely, Ras is able to activate the human ARC promoter region through the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway (75). While specific transcription factors involved in ARC transcription have yet to be discovered, binding sites for transcription factors of the ETS family are present on the ARC promoter region (75). The production of ETS transcription factors has been shown to be activated by Ras/MEK/ERK signalling and the resulting transcription factors have been reported to be involved in carcinogenesis (75–77). Specifically, these transcription factors are involved in the promotion of genes that play a role in the malignant transformation of cells, including growth factor, cell cycle, and apoptotic genes (76). Additionally, this family of transcription factors has been associated with the progression of cancer through the promotion of genes related to invasion and metastasis (76). Ras signalling through the MEK/ERK and the PI3K pathways is also important to ARC transcription and protein levels in OCI3-AML human AML cells (74). Furthermore, the activation of the NIK/MAP3K14 non-canonical NFκB signalling pathway results in an increase in ARC protein levels in human AML cells (78). Interestingly, deregulation of this pathway is associated with lymphoid malignancies (79). Moreover, hypoxia inducing factor 1-alpha (HIF-1α) binds to the hypoxia response element 2 (HRE2) located on the human ARC promoter region and results

in increases in ARC transcription with hypoxia (80). However, it is important to note that the mouse and rat ARC promoter regions do not contain this HRE2 site (80). While there are multiple factors that have been shown to influence the transcriptional regulation of ARC, there are still many unanswered questions.

3 Apoptosis and Cancer

The balance between cell proliferation and cell death is critical in maintaining healthy tissue (2). Disease states arise when this balance is disrupted (2). Typically, neurological and cardiovascular diseases, such as Alzheimer's and heart failure, are a result of excess apoptosis and subsequent loss of neurons and cardiac muscle (81,82). Conversely, inadequate apoptosis can lead to the accumulation of abnormal cells and the formation of a cancerous tumour (83). In reviews by Hanahan and Weinberg, the dysregulation of apoptosis was characterized as one of the hallmarks of cancer development (84,85).

Cancer is simply defined as a disease that is the result of uncontrolled cell division and the accumulation of mutated or damaged cells (84,85). The development of cancer occurs in multiple steps and is often the result of several mutations that work together to create a malignant cell (84). There are numerous mutations that may occur in a cell that can result in the dysregulation of important cellular functions (84,85). The mutation powerful oncogenes, such as *H-ras* and *c-myc*, transform healthy cells into malignant cells and result in uncontrolled proliferation and growth (86). Normally, the mutations that occur would cause a cell to die, however, in order to allow these mutated cells to proliferate, apoptotic mechanisms are generally affected (83). The mutation of p53 in many cancer types results in the elimination of the DNA damage response, which also supports the accumulation of damaged cells (83,85). Additionally, it has been shown

that anti-apoptotic proteins are highly upregulated in some forms of cancer (83,85). The upregulation of ARC has been reported in cancer cell lines and cancer tissue samples (72,73,87). Specifically, human breast cancer and human colon cancer tissue show an upregulation of ARC compared to benign human breast and colon tissue (72,73). Similarly, ARC protein levels are increased in MCF7 human breast cancer cells compared to MCF10A benign breast cells (75). Additionally, ARC protein levels are increased in grade II and grade IV human brain cancer tissue compared to normal brain tissue (87). ARC is also expressed in other cancer cell lines, including HeLa human cervical cancer cells and DU145 human prostate cancer cells (62).

3.1 ARC and cancer

While ARC is normally expressed at low levels in the majority of tissues, ARC becomes highly upregulated when a tissue becomes cancerous (71–73). This increase in ARC has been associated with resistance to chemotherapy and a poor prognosis (70–72).

In a study by Carter and colleagues, blood and bone marrow samples from AML patients with high ARC protein levels were associated with a poor patient prognosis and a higher likelihood of relapse compared to samples that contained low ARC protein levels (71). Additionally, the study found that ARC protein levels were higher at relapse than the levels at the time of the original diagnosis (71). Together, these results indicate that the prognosis of AML is partially associated with ARC protein levels.

In numerous studies, high ARC protein levels have been shown to be a contributor to chemoresistance. Evidently, when ARC is knocked down in the human AML cell line OCI-AML3, these cells become more sensitive to the chemotherapeutic drug cytosine arabinoside (Ara-C), suggesting that ARC is inhibiting the apoptotic potential of the drug (74). A similar effect was found in HeLa cells, where ARC knockdown increased sensitivity to the chemotherapeutic drug

doxorubicin (DOX) (19). One mechanism by which DOX can induce apoptosis is through the activation of PUMA and subsequent mitochondrial fission (19). ARC is able to bind to PUMA and prevent mitochondrial fission, ultimately preventing DOX-induced cell death in HeLa cells (19). Therefore, the knockdown of ARC may have allowed for this mechanism of action to resume function. Furthermore, siRNA mediated ARC knockdown in U251MG glioblastoma cells increased their sensitivity to the chemotherapeutic drug VM-26 (teniposide) (87). Similarly, Hs578T breast cancer cells (which have a low ARC protein content) treated with DOX had a higher percentage of apoptotic nuclei following treatment compared to Hs578T breast cancer cells that overexpress ARC and were also treated with DOX (72). Overall, the results of these studies support the belief that ARC is responsible for chemoresistance in cancer.

The p53 gene is mutated in the majority of cancers cases and this mutation has been shown to play a significant role in the progression of the disease (88). However, in cases where p53 remains active, such as with MCF7 cells, it becomes highly localized to the cytosol, is no longer present in the nucleus, and therefore cannot play a role in transcription (89). This re-localization of p53 may be due to the binding of ARC to the p53 tetramerization domain and the exposure of a nuclear export signal (61). While it has been shown that the protein Parc may play a role in cytosolic retention of p53, Parc is expressed at very low levels in MCF7 breast cancer cells (61,90). On the other hand, ARC expression is very high in MCF7 cells, showing that ARC may be critical in the nuclear export of p53 in these cells (61). Interestingly, the nuclear localization of ARC has been associated with the presence wild-type p53 in breast cancer (61). Taken together, it is clear that ARC plays an important role in preventing wild-type p53 from acting as a transcription factor to induce apoptosis in cancer.

3.2 Apoptotic-related therapies

Some chemotherapeutic drugs available today are focused on creating cellular stress within the cancer cell and subsequently causing cell death (91). However, if anti-apoptotic proteins, such as ARC, are highly expressed in the cancer cells or other components of apoptotic signalling are altered, the drug is likely to be ineffective (92). Therefore, the dysregulation of apoptosis and its related proteins in malignant cells present obvious targets for cancer treatment (91,92).

DOX is a common chemotherapeutic drug that can cause DNA damage and generate free radicals to induce cell death in cancer cells (93). Normally, these conditions created by DOX would activate apoptotic pathways and ultimately result in cell death, however, many cancers are resistant to DOX (19,70). One possible reason for this resistance may be an increase in anti-apoptotic proteins, including ARC (19,70). It has been shown that DOX can decrease ARC protein levels and kill malignant cells, but the high dosage can damage other tissues (19,94).

Researchers are beginning to investigate the effectiveness of combination therapy in the treatment of cancer (95). Since the increase in anti-apoptotic proteins appears to play a significant role in chemoresistance, inhibiting the actions of these proteins would clearly be beneficial to cancer therapy (92,95). Recently, a BCL-2 protein interaction inhibitor has been shown to be effective in causing apoptosis in clinical trials on its own (95). This type of drug can potentially be used in combination with other chemotherapeutic drugs to increase their effectiveness (95). More specifically, the use of a drug that can decrease anti-apoptotic proteins, such as ARC, in combination with apoptosis-inducing chemotherapeutic agents, such as DOX, may result in apoptosis of malignant cells (95).

4 Reporter Systems

Reporter systems are commonly used in molecular biology to investigate the expression levels or localization of a particular protein of interest (96). The coding sequence of the particular protein is inserted into the reporter plasmid and when this plasmid is transfected into a cell, the protein is made with the addition of the reporter protein (97). Reporter systems are also commonly used for promoter analysis, in which the promoter region of interest is inserted into the reporter plasmid (98). In this case, when the promoter region is activated, the reporter protein is made independently and theoretically corresponds with the amount of gene expression (98). Luciferase and green fluorescent protein (GFP) are common reporter proteins that can be easily measured through luminescent and fluorescent assays (96).

4.1 Green fluorescent protein

GFP is a fluorescent protein originally derived from jellyfish (96,99). GFP fluoresces when exposed to the appropriate wavelength of light and does not require the addition of supplementary agents, allowing for the direct and simple detection of gene expression (96,99). GFP can also provide information regarding the location of a protein of interest in a cell if paired with fluorescent microscopy (99). Since GFP detection does not require the addition of cofactors, analysis can be done on living cells and the same cell set can be used for multiple readings (96,99). However, the auto-fluorescence of the cell presents a barrier to the detection of GFP fluorescence (99). With proteins that are not highly expressed or promoter regions that are not strongly activated, GFP fluorescence may not be bright enough to overcome the auto-fluorescence of the cell and the expression may not be detected (99). Additionally, the half-life of GFP is approximately 26 hours, which could result in fluorescence that is due to a build-up of GFP rather than the production of

the gene of interest (100). Overall, despite its potential drawbacks, GFP is a valuable tool for detecting gene expression due to its ease of detection and the ability for live cell analysis.

4.2 Luciferase

Firefly luciferase is an enzyme that catalyzes the reaction between luciferin, ATP, and oxygen to result in light production (Figure 4) (98). When the luciferase plasmid is activated, the luciferase enzyme is produced, leading to the build-up of luciferase within the cell (96). In order for the luciferase to be released from the cell and catalyze the reaction between luciferin, ATP, and oxygen, the cells must be lysed (96). This may limit the use of luciferase as a reporter, as it is not suitable for multiple readings on the same cell set. Furthermore, the addition of expensive substrates is required for the reaction to occur (101). However, luciferase is still a valuable tool for the detection of gene expression. Given that there is limited background noise due to the bioluminescent nature of the luciferase reaction, it is possible to detect small amounts of light production (100). Additionally, the fact that luciferase is an enzyme allows for the continuous reaction between luciferin, ATP, and oxygen and therefore amplification of the signal. Overall, the sensitivity of the luciferase assay makes luciferase an attractive reporter protein, however, the need for the cells to be lysed and the incorporation of additional substrates can limit its use.

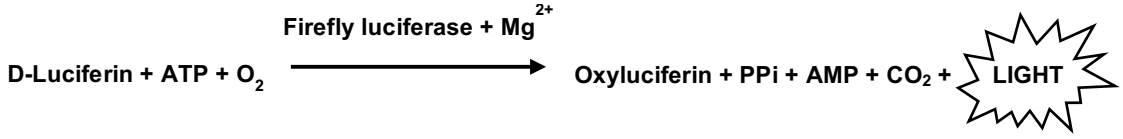


Figure 3: The luciferase reaction. Adapted from Allard, 2008 (102)

Rationale

Cancer is the leading cause of death in Canada (103). While the treatment options available are suitable for some cancers, chemoresistance is becoming a significant problem clinicians are faced with when developing treatment options (92,95).

It has been shown that high ARC expression in some cancers contributes to the resistance to chemotherapeutic drugs due to the apoptotic repressive effects of ARC (70,72,73). In breast cancer, increased ARC levels have been shown to result in a decrease in sensitivity to chemotherapeutic drugs (72). In AML, high ARC protein levels have been shown to be associated with shorter remission durations and decreased overall survival (71). Knockdown of ARC in OCI-AML3 cells resulted in an increased sensitivity to the common AML chemotherapy drug Ara-C (71). Overall, ARC plays a role in chemoresistance in various cancers.

Many chemotherapeutic drugs function to create stress within a cell in order to induce cell death (91). However, if proteins involved in apoptotic pathways are mutated or upregulated, the effectiveness of these drugs can be attenuated (91,95). By discovering a pharmaceutical agent that has the ability to downregulate a powerful anti-apoptotic protein such as ARC, it is thought that apoptotic cell death through chemotherapeutic means will increase (95). Therefore, ARC presents a new therapeutic treatment target.

Purpose and Objectives

ARC is highly upregulated in cancerous tissue and has been associated with chemoresistance, making it an attractive and novel therapeutic target (70–72). The development of a reporter plasmid that contains the ARC promoter region would create a valuable tool to aid in the discovery of potential compounds that alter ARC expression, which could eventually have therapeutic use. Previous research that has analysed the ARC promoter region has used a luciferase-based reporter system (75,80). However, a GFP reporter system presents numerous advantageous over a luciferase reporter system for the use in a high throughput drug screen because GFP requires no additional substrates for detection and does not require the cells to be lysed before analysis (96,99). Therefore, the objective of this thesis project was to develop a novel GFP reporter plasmid to effectively monitor ARC expression.

Hypotheses

It was hypothesized that:

1. GFP fluorescence will correlate with ARC protein levels in MCF7 breast cancer cells.
2. GFP fluorescence will increase with differentiation of C2C12 mouse myoblasts. This increase in GFP fluorescence will correlate with ARC protein levels.

Methods

1 Plasmid development

1.1 pGL3-0.75ARC-EGFP(luc-) and pGL3-0.75ModARC-EGFP(luc-)

The pGL3-0.75ARC luciferase plasmid from Dr. Roger Foo (University of Singapore) was digested to remove the luciferase segment. pGL3-0.75ARC DNA was incubated with the HindIII and XbaI restriction enzymes (Thermo Scientific) and FastDigest green buffer (Thermo Scientific) at 37°C for 2 hours. Following digestion, the mixture was run through a 1% TAE agarose gel to separate the DNA based on size. Ethidium bromide (EtBr) was added to the gel for DNA visualization. The gel was visualised using an ultraviolet transilluminator (TL-2000 Ultraviolet Translinker, UVP) and the band that corresponded to pGL3-0.75ARC(luc-) was cut out. The DNA was subsequently isolated from the agarose gel and purified. First, the piece of agarose gel containing the pGL3-0.75ARC(luc-) DNA was incubated with a chaotropic agent and heated to dissolve the agarose gel. Next, the solution was added to a spin column and centrifuged, which separated the dissolved agarose gel and the DNA. The DNA bound to the membrane within the spin column. To remove impurities, wash buffer was added to the spin column and centrifuged. Finally, the DNA was removed from the membrane using an elution buffer and the pure DNA was collected at the bottom of the tube. This resulted in the pGL3-0.75ARC(luc-) plasmid. The DNA concentration was measured using the NanoDrop 2000 (Thermo Scientific).

The EGFP segment was taken from a pEGFP-N1 plasmid. The EGFP segment could not be directly removed from the pEGFP-N1 plasmid using the HindIII and XbaI restriction enzymes (to allow for insertion into the pGL3-0.75ARC(luc-) plasmid) due to the methylation of the XbaI restriction site. Consequently, polymerase chain reaction (PCR) was used to add a NheI site to the

end of the EGFP segment, since NheI and XbaI restrictions sites have compatible cohesive ends. The following protocol was used for the production of the EGFP segment:

	EGFP from pEGFP-N1 plasmid
Step 1	95°C, 3 min
Step 2	95°C, 30 sec
Step 3	58°C, 30 sec
Step 4	72°C, 1 min
Step 5	Repeat steps 2-4 for 28 cycles
Step 6	72°C, 5 min
Step 7	4°C forever

Table 1: Thermal cycler (Bio-Rad Laboratories) protocol for the production of the EGFP segment.

The PCR product was subsequently run through a 1% TAE agarose gel and the EGFP band was cut out. The DNA was subsequently isolated from the agarose gel and purified. First, the piece of agarose gel containing the PCR product DNA was incubated with a chaotropic agent and heated to dissolve the agarose gel. Next, the solution was added to a spin column and centrifuged, which separated the dissolved agarose gel and the DNA. The DNA bound to the membrane within the spin column. To remove impurities, wash buffer was added to the spin column and centrifuged. Finally, the DNA was removed from the membrane using an elution buffer and the pure DNA was collected at the bottom of the tube. This resulted in the PCR-EGFP segment. The DNA concentration was measured using the NanoDrop 2000 (Thermo Scientific).

Next, the PCR-EGFP segment was ligated into a pGEM plasmid. The pGEM plasmid and the PCR-EGFP segment were incubated with T4 ligase and T4 ligase buffer for 30 minutes at room temperature or overnight at 4°C. Following incubation, the ligation solution was added to competent cells (New England Biolabs) and placed on ice for 30 minutes. Next, the mixture was heat shocked at 42°C for 25 seconds, then immediately placed on ice for 2 minutes. Super optimal

broth with catabolite repression (SOC) was added to the transformation reaction and incubated at 37°C for 90 minutes while shaking vigorously. Following incubation, 100 μL of the reaction was plated onto a lysogeny broth (LB) agar plate with ampicillin and incubated at 37°C overnight. The next day, the small colonies of bacteria that grew on the plate were picked using a pipette tip and placed into glass test tubes containing 5 mL of LB with ampicillin. The tubes were covered and incubated at 37°C overnight while shaking vigorously. The next day, the cloudy LB was purified. First, the cloudy LB was aliquoted into Eppendorf tubes, up to 5 mL in each tube. Next, the tubes were centrifuged to pellet the bacteria. Once the supernatant was removed, the bacteria was re-suspended in buffers that lysed the bacteria and precipitated the cell debris. The tubes were then centrifuged and the supernatant was added to a spin column. The DNA bound to the membrane within the spin column. To remove impurities, wash buffer was added to the spin column and centrifuged. Finally, the DNA was removed from the membrane using an elution buffer and the pure DNA was collected at the bottom of the tube. This resulted in the pGEM-EGFP plasmid. The DNA concentration was measured using the NanoDrop 2000 (Thermo Scientific).

The pGEM-EGFP plasmid was digested to remove the EGFP segment. pGEM-EGFP DNA was incubated with the HindIII and NheI restriction enzymes (Thermo Scientific) and FastDigest green buffer (Thermo Scientific) at 37°C for 2 hours. Following digestion, the mixture was run through a 1% TAE agarose gel to separate the DNA based on size. EtBr was added to the gel for DNA visualization. The gel was visualised using an ultraviolet transilluminator (TL-2000 Ultraviolet Translinker, UVP) and the band that corresponded to EGFP segment was cut out. The DNA was subsequently isolated from the agarose gel and purified. First, the piece of agarose gel containing the EGFP DNA was incubated with a chaotropic agent and heated to dissolve the agarose gel. Next, the solution was added to a spin column and centrifuged, which separated the

dissolved agarose gel and the DNA. The DNA bound to the membrane within the spin column. To remove impurities, wash buffer was added to the spin column and centrifuged. Finally, the DNA was removed from the membrane using an elution buffer and the pure DNA was collected at the bottom of the tube. This resulted in the EGFP segment. The DNA concentration was measured using the NanoDrop 2000 (Thermo Scientific).

The pGL3-0.75ARC(luc-) plasmid and the EGFP segment were incubated with T4 ligase and T4 ligase buffer for 30 minutes at room temperature or overnight at 4°C. Following incubation, the ligation solution was added to competent cells (New England Biolabs) and placed on ice for 30 minutes. Next, the mixture was heat shocked at 42°C for 25 seconds, then immediately placed on ice for 2 minutes. SOC was added to the transformation reaction and incubated at 37°C for 90 minutes while shaking vigorously. Following incubation, 100 μL of the reaction was plated onto a LB agar plate with ampicillin and incubated at 37°C overnight. The next day, the small colonies of bacteria that grew on the plate were picked using a pipette tip and placed into glass test tubes containing 5 mL of LB with ampicillin. The tubes were covered and incubated at 37°C overnight while shaking vigorously. The next day, the cloudy LB was purified. First, the cloudy LB was aliquoted into Eppendorf tubes, up to 5 mL in each tube. Next, the tubes were centrifuged to pellet the bacteria. Once the supernatant was removed, the bacteria was re-suspended in buffers that will lyse the bacteria and precipitate the cell debris. The tubes were then centrifuged and the supernatant was added to a spin column. The DNA bound to the membrane within the spin column. To remove impurities, wash buffer was added to the spin column and centrifuged. Finally, the DNA was removed from the membrane using an elution buffer and the pure DNA was collected at the bottom of the tube. This resulted in the pGL3-0.75ARC-EGFP(luc-) plasmid. The DNA concentration was measured using the NanoDrop 2000 (Thermo Scientific).

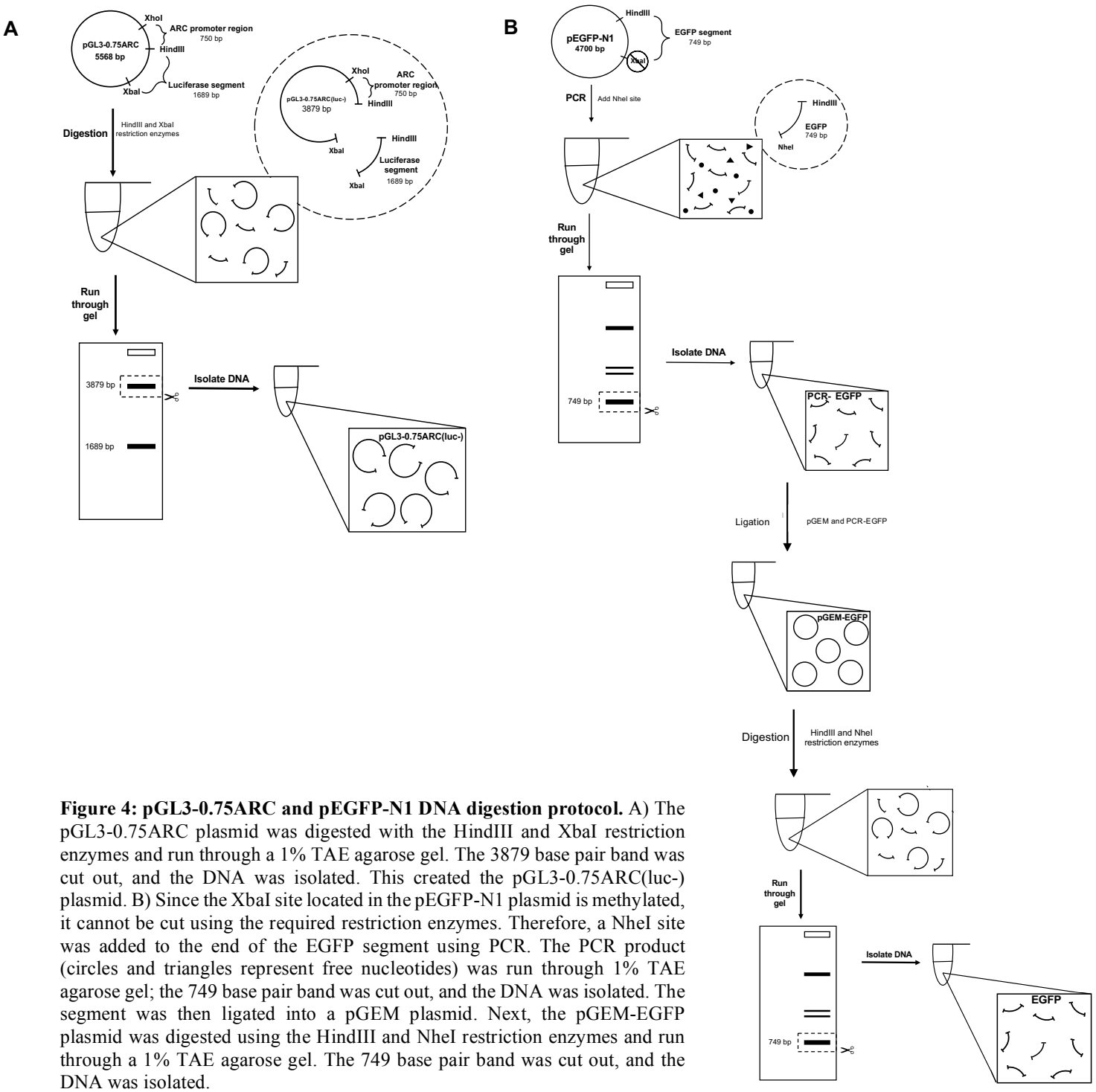


Figure 4: pGL3-0.75ARC and pEGFP-N1 DNA digestion protocol. A) The pGL3-0.75ARC plasmid was digested with the HindIII and XbaI restriction enzymes and run through a 1% TAE agarose gel. The 3879 base pair band was cut out, and the DNA was isolated. This created the pGL3-0.75ARC(luc-) plasmid. B) Since the XbaI site located in the pEGFP-N1 plasmid is methylated, it cannot be cut using the required restriction enzymes. Therefore, a NheI site was added to the end of the EGFP segment using PCR. The PCR product (circles and triangles represent free nucleotides) was run through 1% TAE agarose gel; the 749 base pair band was cut out, and the DNA was isolated. The segment was then ligated into a pGEM plasmid. Next, the pGEM-EGFP plasmid was digested using the HindIII and NheI restriction enzymes and run through a 1% TAE agarose gel. The 749 base pair band was cut out, and the DNA was isolated.

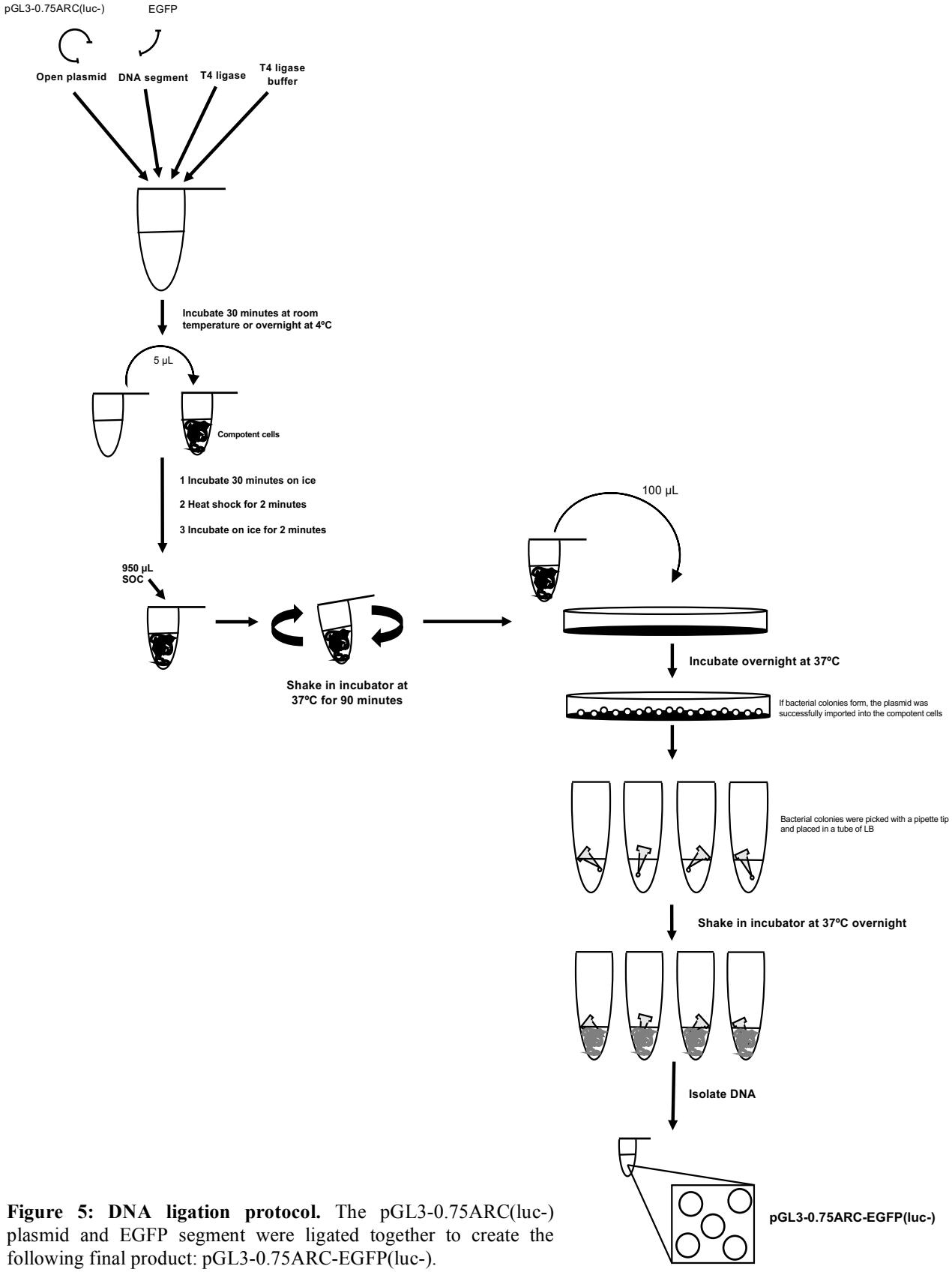


Figure 5: DNA ligation protocol. The pGL3-0.75ARC(luc-) plasmid and EGFP segment were ligated together to create the following final product: pGL3-0.75ARC-EGFP(luc-).

In order to remove a stop codon present in the pGL3-0.75ARC-EGFP(luc-) plasmid just before the EGFP sequence, this region of the plasmid was modified using the following primers: forward, 5'-TAC GCG TGC TAG CCC GGG CTC GAG-3'; reverse, 5'-AAG CTT CCG CTC CTG CGC GTT GCC CAT-3'. The forward primer contains the sequence for a XhoI restriction site and the beginning of the 750 base pair ARC promoter region and the reverse primer contains the sequence for the end for the 750 base pair ARC promoter region and a HindIII restriction site, creating a segment that does not contain the stop codon. The following thermal cycler protocol was used:

	Modified ARC segment
Step 1	94°C, 3 min
Step 2	94°C, 1 min
Step 3	60°C, 1 min
Step 4	72°C, 1 min
Step 5	Repeat steps 2-4 for 34 cycles
Step 6	72°C, 10 min
Step 7	4°C forever

Table 2: Thermal cycler (Bio-Rad Laboratories) protocol for the production of the modified ARC segment.

The PCR product was subsequently run through a 1% TAE agarose gel, and the band corresponding with the modified ARC segment was cut out. The DNA was subsequently isolated from the agarose gel and purified. First, the piece of agarose gel containing the modified ARC segment DNA was incubated with a chaotropic agent and heated to dissolve the agarose gel. Next, the solution was added to a spin column and centrifuged, which separated the dissolved agarose gel and the DNA. The DNA bound to the membrane within the spin column. To remove impurities, wash buffer was added to the spin column and centrifuged. Finally, the DNA was removed from the membrane using an elution buffer and the pure DNA was collected at the bottom of the tube.

This resulted in the PCR-modified ARC (PCR-ModARC) segment. The DNA concentration was measured using the NanoDrop 2000 (Thermo Scientific).

Next, the PCR-ModARC segment was ligated into a pGEM plasmid. The pGEM plasmid and the PCR-ModARC segment were incubated with T4 ligase and T4 ligase buffer for 30 minutes at room temperature or overnight at 4°C. Following incubation, the ligation solution was added to competent cells (New England Biolabs) and placed on ice for 30 minutes. Next, the mixture was heat shocked at 42°C for 25 seconds, then immediately placed on ice for 2 minutes. SOC was added to the transformation reaction and incubated at 37°C for 90 minutes while shaking vigorously. Following incubation, 100 μL of the reaction was plated onto a LB agar plate with ampicillin and incubated at 37°C overnight. The next day, the small colonies of bacteria that grew on the plate were picked using a pipette tip and placed into glass test tubes containing 5 mL of LB with ampicillin. The tubes were covered and incubated at 37°C overnight while shaking vigorously. The next day, the cloudy LB was purified. First, the cloudy LB was aliquoted into Eppendorf tubes, up to 5 mL in each tube. Next, the tubes were centrifuged to pellet the bacteria. Once the supernatant was removed, the bacteria was re-suspended in buffers that lysed the bacteria and precipitated the cell debris. The tubes were then centrifuged and the supernatant was added to a spin column. The DNA bound to the membrane within the spin column. To remove impurities, wash buffer was added to the spin column and centrifuged. Finally, the DNA was removed from the membrane using an elution buffer and the pure DNA was collected at the bottom of the tube. This resulted in the pGEM-ModARC plasmid. The DNA concentration was measured using the NanoDrop 2000 (Thermo Scientific).

The pGEM-ModARC plasmid was digested to remove the ModARC segment. pGEM-ModARC DNA was incubated with the XhoI and HindIII restriction enzymes (Thermo

Scientific) and FastDigest green buffer (Thermo Scientific) at 37°C for 2 hours. Following digestion, the mixture was run through a 1% TAE agarose gel to separate the DNA based on size. EtBr was added to the gel for DNA visualization. The gel was visualised using an ultraviolet transilluminator (TL-2000 Ultraviolet Translinker, UVP) and the band that corresponded to ModARC segment was cut out. The DNA was subsequently isolated from the agarose gel and purified. First, the piece of agarose gel containing the ModARC DNA was incubated with a chaotropic agent and heated to dissolve the agarose gel. Next, the solution was added to a spin column and centrifuged, which separated the dissolved agarose gel and the DNA. The DNA bound to the membrane within the spin column. To remove impurities, wash buffer was added to the spin column and centrifuged. Finally, the DNA was removed from the membrane using an elution buffer and the pure DNA was collected at the bottom of the tube. This resulted in the ModARC segment. The DNA concentration was measured using the NanoDrop 2000 (Thermo Scientific).

The pGL3-0.75ARC-EGFP(luc-) luciferase was digested to remove the segment containing the stop codon. pGL3-0.75ARC-EGFP(luc-) DNA was incubated with the XhoI and HindIII restriction enzymes (Thermo Scientific) and FastDigest green buffer (Thermo Scientific) at 37°C for 2 hours. Following digestion, the mixture was run through a 1% TAE agarose gel to separate the DNA based on size. EtBr was added to the gel for DNA visualization. The gel was visualised using an ultraviolet transilluminator (TL-2000 Ultraviolet Translinker, UVP) and the band that corresponded to pGL3-EGFP(luc-) was cut out. The DNA was subsequently isolated from the agarose gel and purified. First, the piece of agarose gel containing the pGL3-EGFP(luc-) DNA was incubated with a chaotropic agent and heated to dissolve the agarose gel. Next, the solution was added to a spin column and centrifuged, which separated the dissolved agarose gel and the DNA. The DNA bound to the membrane within the spin column. To remove impurities, wash

buffer was added to the spin column and centrifuged. Finally, the DNA was removed from the membrane using an elution buffer and the pure DNA was collected at the bottom of the tube. This resulted in the open pGL3-EGFP(luc-) plasmid. The DNA concentration was measured using the NanoDrop 2000 (Thermo Scientific).

The open pGL3-EGFP(luc-) plasmid and ModARC segment were incubated with T4 ligase and T4 ligase buffer for 30 minutes at room temperature or overnight at 4°C. Following incubation, the ligation solution was added to competent cells (New England Biolabs) and placed on ice for 30 minutes. Next, the mixture was heat shocked at 42°C for 25 seconds, then immediately placed on ice for 2 minutes. SOC was added to the transformation reaction and incubated at 37°C for 90 minutes while shaking vigorously. Following incubation, 100 μL of the reaction was plated onto a LB agar plate with ampicillin and incubated at 37°C overnight. The next day, the small colonies of bacteria that grew on the plate were picked using a pipette tip and placed into glass test tubes containing 5 mL of LB with ampicillin. The tubes were covered and incubated at 37°C overnight while shaking vigorously. The next day, the cloudy LB was purified. First, the cloudy LB was aliquoted into Eppendorf tubes, up to 5 mL in each tube. Next, the tubes were centrifuged to pellet the bacteria. Once the supernatant was removed, the bacteria was re-suspended in buffers that will lyse the bacteria and precipitate the cell debris. The tubes were then centrifuged and the supernatant was added to a spin column. The DNA bound to the membrane within the spin column. To remove impurities, wash buffer was added to the spin column and centrifuged. Finally, the DNA was removed from the membrane using an elution buffer and the pure DNA was collected at the bottom of the tube. This resulted in the pGL3-0.75ModARC-EGFP(luc-) plasmid. The DNA concentration was measured using the NanoDrop 2000 (Thermo Scientific).

1.2 pGL3-1.005ARC-EGFP(luc-)

The pGL3-1.005ARC luciferase plasmid from Dr. Olga Razorenova (University of California, Irvine) was digested to remove the luciferase segment. pGL3-1.005ARC DNA was incubated with the HindIII and XbaI restriction enzymes (Thermo Scientific) and FastDigest green buffer (Thermo Scientific) at 37°C for 2 hours. Following digestion, the mixture was run through a 1% TAE agarose gel to separate the DNA based on size. EtBr was added to the gel for DNA visualization. The gel was visualised using an ultraviolet transilluminator (TL-2000 Ultraviolet Translinker, UVP) and the band that corresponded to pGL3-1.005ARC(luc-) was cut out. The DNA was subsequently isolated from the agarose gel and purified. First, the piece of agarose gel containing the pGL3-1.005ARC(luc-) DNA was incubated with a chaotropic agent and heated to dissolve the agarose gel. Next, the solution was added to a spin column and centrifuged, which separated the dissolved agarose gel and the DNA. The DNA bound to the membrane within the spin column. To remove impurities, wash buffer was added to the spin column and centrifuged. Finally, the DNA was removed from the membrane using an elution buffer and the pure DNA was collected at the bottom of the tube. This resulted in the pGL3-1.005ARC(luc-) plasmid. The DNA concentration was measured using the NanoDrop 2000 (Thermo Scientific).

The EGFP segment was taken from a pEGFP-N1 plasmid. The EGFP segment could not be directly removed from the pEGFP-N1 plasmid using the HindIII and XbaI restriction enzymes (to allow for insertion into the pGL3-1.005ARC(luc-) plasmid) due to the methylation of the XbaI restriction site. Consequently, polymerase chain reaction (PCR) was used to add a NheI site to the end of the EGFP segment, since NheI and XbaI restrictions sites have compatible cohesive ends. The following protocol was used for the production of the EGFP segment:

	EGFP from pEGFP-N1 plasmid
Step 1	95°C, 3 min
Step 2	95°C, 30 sec
Step 3	58°C, 30 sec
Step 4	72°C, 1 min
Step 5	Repeat steps 2-4 for 28 cycles
Step 6	72°C, 5 min
Step 7	4°C forever

Table 3: Thermal cycler (Bio-Rad Laboratories) protocol for the production of the EGFP segment.

The PCR product was subsequently run through a 1% TAE agarose gel, and the EGFP band was cut out. The DNA was subsequently isolated from the agarose gel and purified. First, the piece of agarose gel containing the PCR product DNA was incubated with a chaotropic agent and heated to dissolve the agarose gel. Next, the solution was added to a spin column and centrifuged, which separated the dissolved agarose gel and the DNA. The DNA bound to the membrane within the spin column. To remove impurities, wash buffer was added to the spin column and centrifuged. Finally, the DNA was removed from the membrane using an elution buffer and the pure DNA was collected at the bottom of the tube. This resulted in the PCR-EGFP segment. The DNA concentration was measured using the NanoDrop 2000 (Thermo Scientific).

Next, the PCR-EGFP segment was ligated into a pGEM plasmid. The pGEM plasmid and the PCR-EGFP segment were incubated with T4 ligase and T4 ligase buffer for 30 minutes at room temperature or overnight at 4°C. Following incubation, the ligation solution was added to competent cells (New England Biolabs) and placed on ice for 30 minutes. Next, the mixture was heat shocked at 42°C for 25 seconds, then immediately placed on ice for 2 minutes. SOC was added to the transformation reaction and incubated at 37°C for 90 minutes while shaking vigorously. Following incubation, 100 μL of the reaction was plated onto a LB agar plate with

ampicillin and incubated at 37°C overnight. The next day, the small colonies of bacteria that grew on the plate were picked using a pipette tip and placed into glass test tubes containing 5 mL of LB with ampicillin. The tubes were covered and incubated at 37°C overnight while shaking vigorously. The next day, the cloudy LB was purified. First, the cloudy LB was aliquoted into Eppendorf tubes, up to 5 mL in each tube. Next, the tubes were centrifuged to pellet the bacteria. Once the supernatant was removed, the bacteria was re-suspended in buffers that lysed the bacteria and precipitated the cell debris. The tubes were then centrifuged and the supernatant was added to a spin column. The DNA bound to the membrane within the spin column. To remove impurities, wash buffer was added to the spin column and centrifuged. Finally, the DNA was removed from the membrane using an elution buffer and the pure DNA was collected at the bottom of the tube. This resulted in the pGEM-EGFP plasmid. The DNA concentration was measured using the NanoDrop 2000 (Thermo Scientific).

The pGEM-EGFP plasmid was digested to remove the EGFP segment. pGEM-EGFP DNA was incubated with the HindIII and NheI restriction enzymes (Thermo Scientific) and FastDigest green buffer (Thermo Scientific) at 37°C for 2 hours. Following digestion, the mixture was run through a 1% TAE agarose gel to separate the DNA based on size. EtBr was added to the gel for DNA visualization. The gel was visualised using an ultraviolet transilluminator (TL-2000 Ultraviolet Translinker, UVP) and the band that corresponded to EGFP segment was cut out. The DNA was subsequently isolated from the agarose gel and purified. First, the piece of agarose gel containing the EGFP DNA was incubated with a chaotropic agent and heated to dissolve the agarose gel. Next, the solution was added to a spin column and centrifuged, which separated the dissolved agarose gel and the DNA. The DNA bound to the membrane within the spin column. To remove impurities, wash buffer was added to the spin column and centrifuged. Finally, the DNA

was removed from the membrane using an elution buffer and the pure DNA was collected at the bottom of the tube. This resulted in the EGFP segment. The DNA concentration was measured using the NanoDrop 2000 (Thermo Scientific).

The pGL3-1.005ARC(luc-) plasmid and the EGFP segment were incubated with T4 ligase and T4 ligase buffer for 30 minutes at room temperature or overnight at 4°C. Following incubation, the ligation solution was added to competent cells (New England Biolabs) and placed on ice for 30 minutes. Next, the mixture was heat shocked at 42°C for 25 seconds, then immediately placed on ice for 2 minutes. SOC was added to the transformation reaction and incubated at 37°C for 90 minutes while shaking vigorously. Following incubation, 100 μL of the reaction was plated onto a LB agar plate with ampicillin and incubated at 37°C overnight. The next day, the small colonies of bacteria that grew on the plate were picked using a pipette tip and placed into glass test tubes containing 5 mL of LB with ampicillin. The tubes were covered and incubated at 37°C overnight while shaking vigorously. The next day, the cloudy LB was purified. First, the cloudy LB was aliquoted into Eppendorf tubes, up to 5 mL in each tube. Next, the tubes were centrifuged to pellet the bacteria. Once the supernatant was removed, the bacteria was re-suspended in buffers that will lyse the bacteria and precipitate the cell debris. The tubes were then centrifuged and the supernatant was added to a spin column. The DNA bound to the membrane within the spin column. To remove impurities, wash buffer was added to the spin column and centrifuged. Finally, the DNA was removed from the membrane using an elution buffer and the pure DNA was collected at the bottom of the tube. This resulted in the pGL3-1.005ARC-EGFP(luc-) plasmid. The DNA concentration was measured using the NanoDrop 2000 (Thermo Scientific).

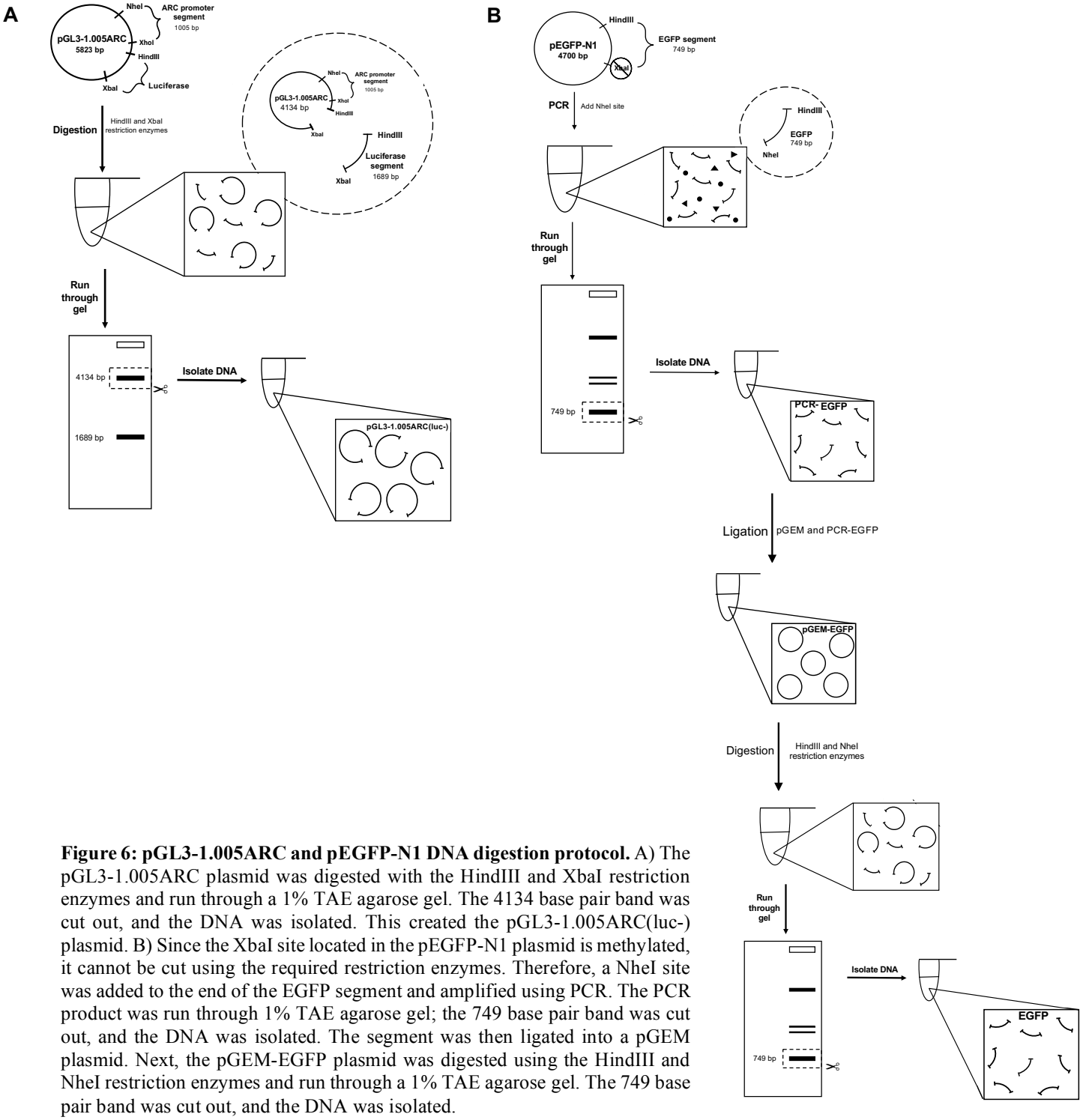


Figure 6: pGL3-1.005ARC and pEGFP-N1 DNA digestion protocol. A) The pGL3-1.005ARC plasmid was digested with the HindIII and XbaI restriction enzymes and run through a 1% TAE agarose gel. The 4134 base pair band was cut out, and the DNA was isolated. This created the pGL3-1.005ARC(luc-) plasmid. B) Since the XbaI site located in the pEGFP-N1 plasmid is methylated, it cannot be cut using the required restriction enzymes. Therefore, a NheI site was added to the end of the EGFP segment and amplified using PCR. The PCR product was run through 1% TAE agarose gel; the 749 base pair band was cut out, and the DNA was isolated. The segment was then ligated into a pGEM plasmid. Next, the pGEM-EGFP plasmid was digested using the HindIII and NheI restriction enzymes and run through a 1% TAE agarose gel. The 749 base pair band was cut out, and the DNA was isolated.

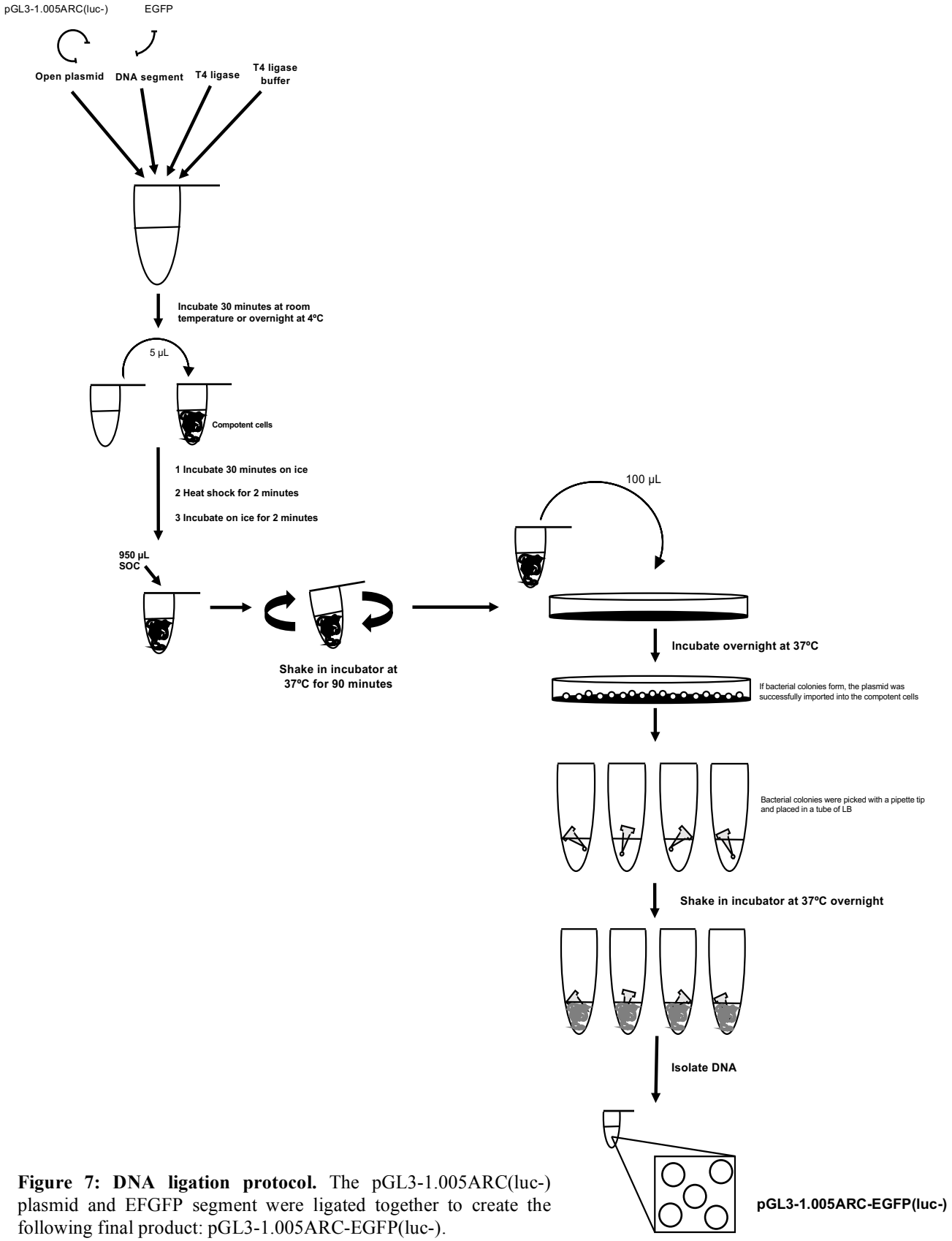


Figure 7: DNA ligation protocol. The pGL3-1.005ARC(luc-) plasmid and EFGFP segment were ligated together to create the following final product: pGL3-1.005ARC-EGFP(luc-).

1.3 pEGFP-N1-1.005ARC(CMV-)

To remove the CMV promoter from the pEGFP-N1 plasmid, AseI and BamHI restriction sites were used. Therefore, in order for the 1005 base pair ARC segment to be added to the EGFP-N1 plasmid, new restriction sites were required. The new restriction sites, AseI and BglII, were added to the beginning and end of the 1005 base pair ARC segment, respectively, using PCR. BglII was used because there is a BamHI site within the 1005 base pair ARC segment. BglII and BamHI have compatible cohesive ends. The following primers were used: 5'-TAA GCA ATT AAT AAG GGG CTT GGA ACC AGT CC-3'; reverse, 5'-TGC TTA AGA TCT GTG CGA CTG CAC GGA TTT TC-3'. The forward primer contains the sequence for a AseI restriction site and the beginning of the 1005 base pair ARC segment and the reverse primer contains the sequence for the end of the 1005 base pair ARC segment and a BglII restriction site. The following thermal cycler protocol was used:

ARC segment from pGL3-1.005ARC
94°C, 2 min
94°C, 30 sec
60°C, 60 sec
72°C, 2 min
Repeat steps 2-4 for 34 cycles
72°C, 5 min
12°C forever

Table 4: Thermal cycler (Bio-Rad Laboratories) protocol for the production of the 1005 base pair region of the ARC promoter with AseI and BglII restriction sites.

The PCR product was digested using AseI and BglII restriction enzymes (Thermo Scientific/New England Biolabs) and FastDigest green buffer (Thermo Scientific) at 37°C for 2 hours. Following digestion, the mixture was run through a 1% TAE agarose gel, and the band

corresponding to the 1005 base pair ARC segment was cut out. The DNA was subsequently isolated from the agarose gel and purified. First, the piece of agarose gel containing the 1005 base pair ARC segment DNA was incubated with a chaotropic agent and heated to dissolve the agarose gel. Next, the solution was added to a spin column and centrifuged, which separated the dissolved agarose gel and the DNA. The DNA bound to the membrane within the spin column. To remove impurities, wash buffer was added to the spin column and centrifuged. Finally, the DNA was removed from the membrane using an elution buffer and the pure DNA was collected at the bottom of the tube. This resulted in the 1005 base pair ARC segment containing AseI and BglII restriction sites at the beginning and end of the segment, respectively. The DNA concentration was measured using the NanoDrop 2000 (Thermo Scientific).

The pEGFP-N1 plasmid was digested to remove the CMV promoter. pEGFP-N1 DNA was incubated with the AseI and BamHI restriction enzymes (Thermo Scientific/New England Biolabs) and FastDigest green buffer (Thermo Scientific) at 37°C for 2 hours. Following digestion, the mixture was run through a 1% TAE agarose gel to separate the DNA based on size. The gel was visualised using an ultraviolet transilluminator (TL-2000 Ultraviolet Translinker, UVP) and the band that corresponded to the pEGFP-N1(CMV-) plasmid was cut out. The DNA was subsequently isolated from the agarose gel and purified. First, the piece of agarose gel containing the pEGFP-N1(CMV-) DNA was incubated with a chaotropic agent and heated to dissolve the agarose gel. Next, the solution was added to a spin column and centrifuged, which separated the dissolved agarose gel and the DNA. The DNA bound to the membrane within the spin column. To remove impurities, wash buffer was added to the spin column and centrifuged. Finally, the DNA was removed from the membrane using an elution buffer and the pure DNA was collected at the

bottom of the tube. This resulted in the pEGFP-N1(CMV-) plasmid. The DNA concentration was measured using the NanoDrop 2000 (Thermo Scientific).

The pEGFP-N1(CMV-) plasmid and the 1005 base pair ARC segment were incubated with T4 ligase and T4 ligase buffer for 30 minutes at room temperature or overnight at 4°C. Following incubation, the ligation solution was added to competent cells (New England Biolabs) and placed on ice for 30 minutes. Next, the mixture was heat shocked at 42°C for 25 seconds, then immediately placed on ice for 2 minutes. SOC was added to the transformation reaction and incubated at 37°C for 90 minutes while shaking vigorously. Following incubation, 100 μL of the reaction was plated onto a LB agar plate with kanamycin and incubated at 37°C overnight. The next day, the small colonies of bacteria that grew on the plate were picked using a pipette tip and placed into glass test tubes containing 5 mL of LB with kanamycin. The tubes were covered and incubated at 37°C overnight while shaking vigorously. The next day, the cloudy LB was purified. First, the cloudy LB was aliquoted into Eppendorf tubes, up to 5 mL in each tube. Next, the tubes were centrifuged to pellet the bacteria. Once the supernatant was removed, the bacteria was re-suspended in buffers that will lyse the bacteria and precipitate the cell debris. The tubes were then centrifuged and the supernatant was added to a spin column. The DNA bound to the membrane within the spin column. To remove impurities, wash buffer was added to the spin column and centrifuged. Finally, the DNA was removed from the membrane using an elution buffer and the pure DNA was collected at the bottom of the tube. This resulted in the pEGFP-N1-1.005ARC(CMV-) plasmid. The DNA concentration was measured using the NanoDrop 2000 (Thermo Scientific).

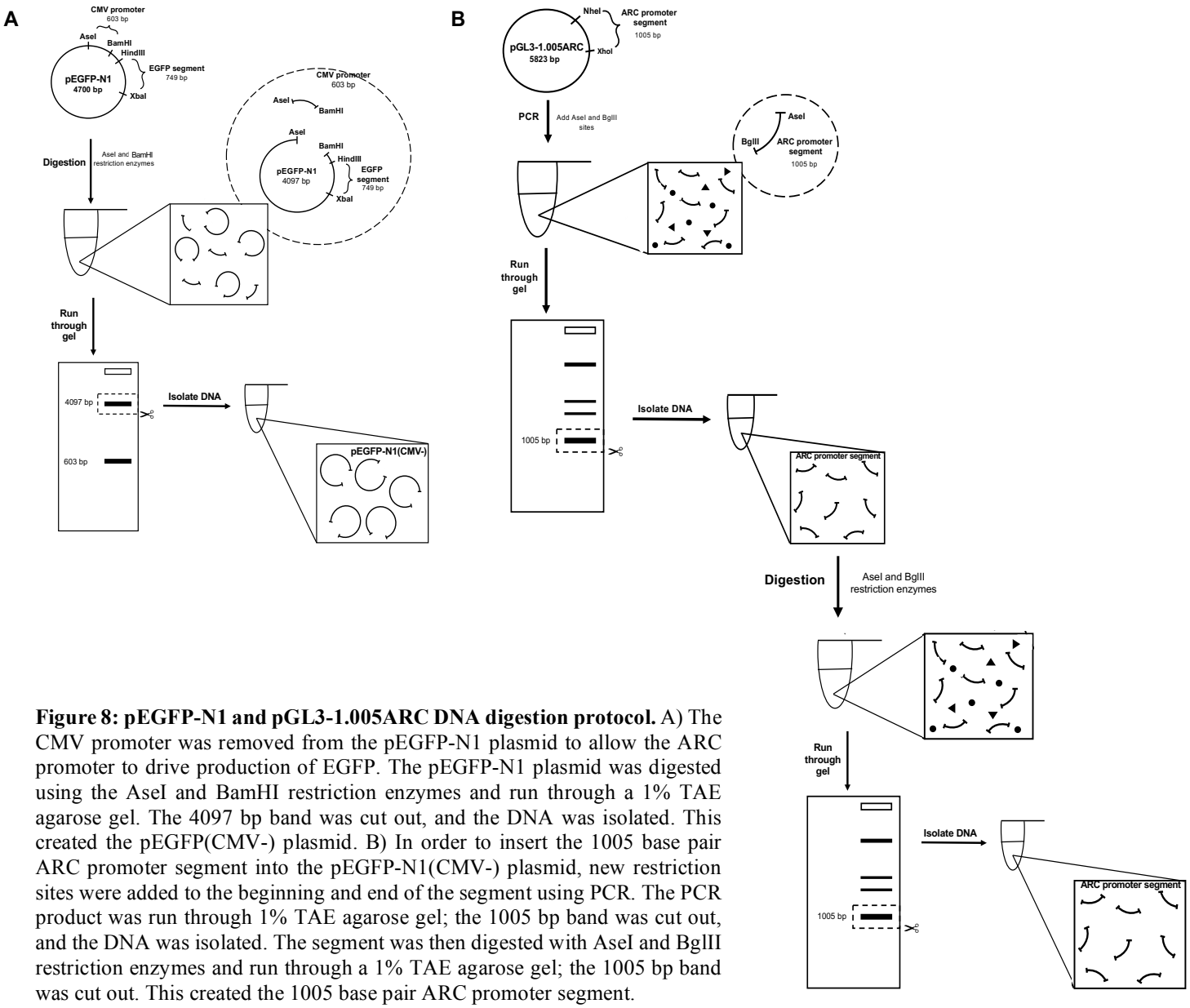


Figure 8: pEGFP-N1 and pGL3-1.005ARC DNA digestion protocol. A) The CMV promoter was removed from the pEGFP-N1 plasmid to allow the ARC promoter to drive production of EGFP. The pEGFP-N1 plasmid was digested using the AseI and BamHI restriction enzymes and run through a 1% TAE agarose gel. The 4097 bp band was cut out, and the DNA was isolated. This created the pEGFP(CMV-) plasmid. B) In order to insert the 1005 base pair ARC promoter segment into the pEGFP-N1(CMV-) plasmid, new restriction sites were added to the beginning and end of the segment using PCR. The PCR product was run through 1% TAE agarose gel; the 1005 bp band was cut out, and the DNA was isolated. The segment was then digested with AseI and BglII restriction enzymes and run through a 1% TAE agarose gel; the 1005 bp band was cut out. This created the 1005 base pair ARC promoter segment.

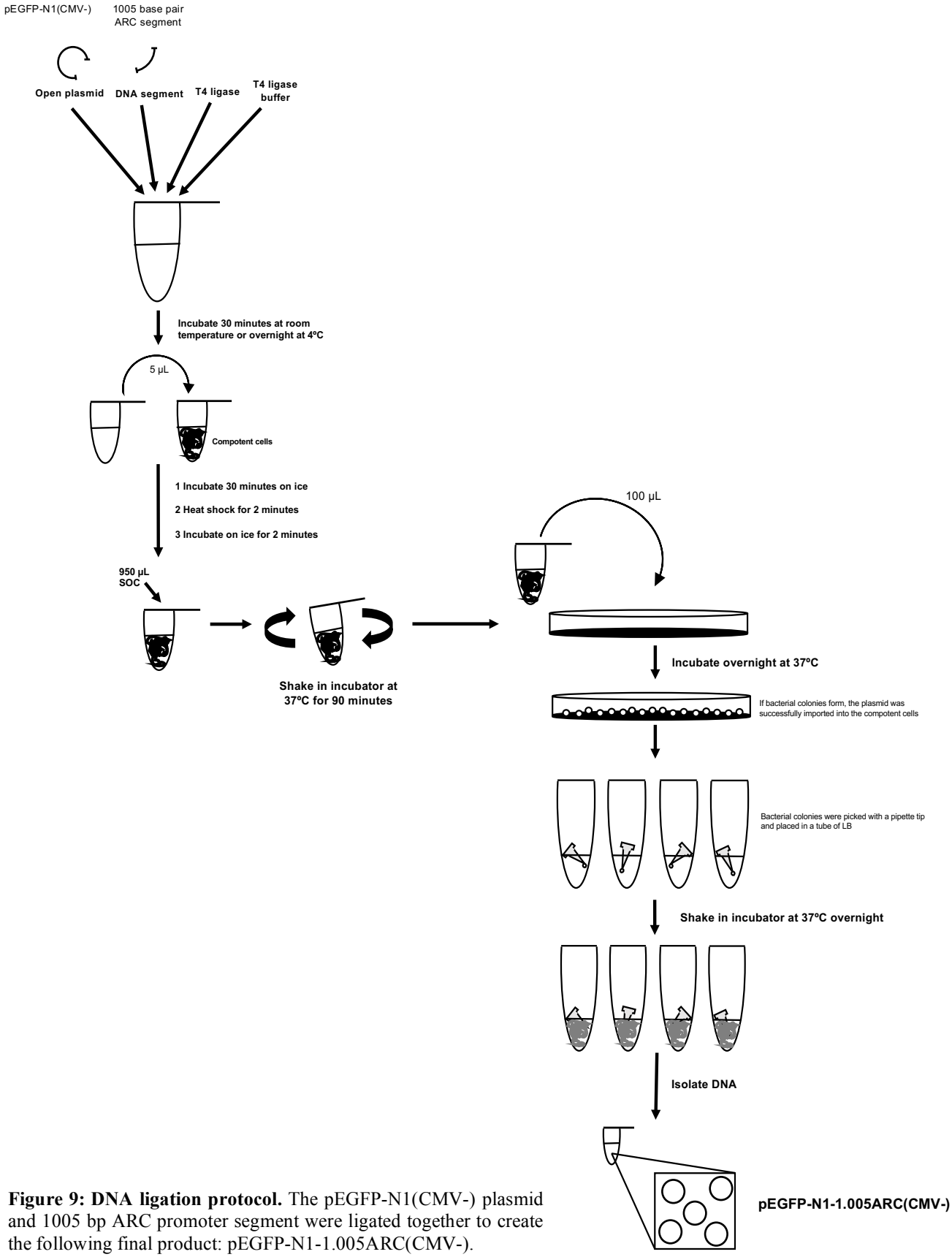


Figure 9: DNA ligation protocol. The pEGFP-N1(CMV-) plasmid and 1005 bp ARC promoter segment were ligated together to create the following final product: pEGFP-N1-1.005ARC(CMV-).

1.4 Promoterless controls

The promoterless, negative control plasmid was made by digesting the pGL3-0.75ARC-EGFP(luc-) plasmid using the Kpn-1 restriction enzyme, since there are Kpn-1 restriction sites flanking the 750 base pair ARC promoter region. pGL3-0.75ARC-EGFP(luc-) DNA was incubated with the Kpn-1 restriction enzyme (Thermo Scientific) and FastDigest green buffer (Thermo Scientific) at 37°C for 2 hours. Following digestion, the mixture was run through a 1% TAE agarose gel to separate the DNA based on size. The gel was visualised using an ultraviolet transilluminator (TL-2000 Ultraviolet Translinker, UVP) and the band that corresponded to the pGL3-EGFP(luc-) plasmid was cut out. The DNA was subsequently isolated from the agarose gel and purified. First, the piece of agarose gel containing the pGL3-EGFP(luc-) DNA was incubated with a chaotropic agent and heated to dissolve the agarose gel. Next, the solution was added to a spin column and centrifuged, which separated the dissolved agarose gel and the DNA. The DNA bound to the membrane within the spin column. To remove impurities, wash buffer was added to the spin column and centrifuged. Finally, the DNA was removed from the membrane using an elution buffer and the pure DNA was collected at the bottom of the tube. This resulted in the open pGL3-EGFP(luc-) plasmid. The DNA concentration was measured using the NanoDrop 2000 (Thermo Scientific).

Following digestion, the open ends of the plasmid were ligated back together. The pGL3-EGFP(luc-) plasmid was incubated with T4 ligase and T4 ligase buffer for 30 minutes at room temperature or overnight at 4°C. Following incubation, the ligation solution was added to competent cells (New England Biolabs) and placed on ice for 30 minutes. Next, the mixture was heat shocked at 42°C for 25 seconds, then immediately placed on ice for 2 minutes. SOC was added to the transformation reaction and incubated at 37°C for 90 minutes while shaking

vigorously. Following incubation, 100 μL of the reaction was plated onto a LB agar plate with ampicillin and incubated at 37°C overnight. The next day, the small colonies of bacteria that grew on the plate were picked using a pipette tip and placed into glass test tubes containing 5 mL of LB with ampicillin. The tubes were covered and incubated at 37°C overnight while shaking vigorously. The next day, the cloudy LB was purified. First, the cloudy LB was aliquoted into Eppendorf tubes, up to 5 mL in each tube. Next, the tubes were centrifuged to pellet the bacteria. Once the supernatant was removed, the bacteria was re-suspended in buffers that will lyse the bacteria and precipitate the cell debris. The tubes were then centrifuged and the supernatant was added to a spin column. The DNA bound to the membrane within the spin column. To remove impurities, wash buffer was added to the spin column and centrifuged. Finally, the DNA was removed from the membrane using an elution buffer and the pure DNA was collected at the bottom of the tube. This resulted in the pGL3-EGFP(luc-) plasmid. The DNA concentration was measured using the NanoDrop 2000 (Thermo Scientific).

To make the promoterless, negative control plasmid for the luciferase assay, the pGL3-0.75ARC plasmid was digested with the Kpn-1 restriction enzyme, since there are Kpn-1 restriction sites flanking the 750 base pair ARC promoter region. pGL3-0.75ARC DNA was incubated with the Kpn-1 restriction enzyme (Thermo Scientific) and FastDigest green buffer (Thermo Scientific) at 37°C for 2 hours. Following digestion, the mixture was run through a 1% TAE agarose gel to separate the DNA based on size. The gel was visualised using an ultraviolet transilluminator (TL-2000 Ultraviolet Translinker, UVP) and the band that corresponded to the pGL3 plasmid was cut out. The DNA was subsequently isolated from the agarose gel and purified. First, the piece of agarose gel containing the pGL3 DNA was incubated with a chaotropic agent and heated to dissolve the agarose gel. Next, the solution was added to a spin column and

centrifuged, which separated the dissolved agarose gel and the DNA. The DNA bound to the membrane within the spin column. To remove impurities, wash buffer was added to the spin column and centrifuged. Finally, the DNA was removed from the membrane using an elution buffer and the pure DNA was collected at the bottom of the tube. This resulted in the open pGL3 plasmid. The DNA concentration was measured using the NanoDrop 2000 (Thermo Scientific).

Following digestion, the open ends of the plasmid were ligated back together. The pGL3 plasmid was incubated with T4 ligase and T4 ligase buffer for 30 minutes at room temperature or overnight at 4°C. Following incubation, the ligation solution was added to competent cells (New England Biolabs) and placed on ice for 30 minutes. Next, the mixture was heat shocked at 42°C for 25 seconds, then immediately placed on ice for 2 minutes. SOC was added to the transformation reaction and incubated at 37°C for 90 minutes while shaking vigorously. Following incubation, 100 μL of the reaction was plated onto a LB agar plate with ampicillin and incubated at 37°C overnight. The next day, the small colonies of bacteria that grew on the plate were picked using a pipette tip and placed into glass test tubes containing 5 mL of LB with ampicillin. The tubes were covered and incubated at 37°C overnight while shaking vigorously. The next day, the cloudy LB was purified. First, the cloudy LB was aliquoted into Eppendorf tubes, up to 5 mL in each tube. Next, the tubes were centrifuged to pellet the bacteria. Once the supernatant was removed, the bacteria was re-suspended in buffers that will lyse the bacteria and precipitate the cell debris. The tubes were then centrifuged and the supernatant was added to a spin column. The DNA bound to the membrane within the spin column. To remove impurities, wash buffer was added to the spin column and centrifuged. Finally, the DNA was removed from the membrane using an elution buffer and the pure DNA was collected at the bottom of the tube. This resulted in the pGL3 plasmid. The DNA concentration was measured using the NanoDrop 2000 (Thermo Scientific).

Since there were no compatible restriction sites in the pEGFP-N1 plasmid, the promoterless, negative control plasmid (pEGFP-N1(CMV-)) was made using scramble oligonucleotides containing AseI and BglII restriction sites (forward, 5'-TAA TGC ACT ACC AGA GCT AAC TCA-3'; reverse, 5'-GAT CTG AGT TAG CTC TGG TAG TGC AT-3'). The following protocol was used to anneal the sequences:

	Scram DNA
Step 1	37°C, 30 min
Step 2	95°C, 5 min
Step 3	25°C, 10 sec
Step 4	Rate 0.1
Step 5	25°C forever

Table 5: Thermal cycler (Bio-Rad Laboratories) protocol for the production of the scramble oligonucleotide.

The scramble segment was subsequently ligated into the open pEGFP-N1(CMV-) plasmid. The open pEGFP-N1(CMV-) plasmid and oligonucleotide segment were incubated with T4 ligase and T4 ligase buffer for 30 minutes at room temperature or overnight at 4°C. Following incubation, the ligation solution was added to competent cells (New England Biolabs) and placed on ice for 30 minutes. Next, the mixture was heat shocked at 42°C for 25 seconds, then immediately placed on ice for 2 minutes. SOC was added to the transformation reaction and incubated at 37°C for 90 minutes while shaking vigorously. Following incubation, 100 μL of the reaction was plated onto a LB agar plate with kanamycin and incubated at 37°C overnight. The next day, the small colonies of bacteria that grew on the plate were picked using a pipette tip and placed into glass test tubes containing 5 mL of LB with kanamycin. The tubes were covered and incubated at 37°C overnight while shaking vigorously. The next day, the cloudy LB was purified. First, the cloudy LB was aliquoted into Eppendorf tubes, up to 5 mL in each tube. Next, the tubes were centrifuged to pellet

the bacteria. Once the supernatant was removed, the bacteria was re-suspended in buffers that will lyse the bacteria and precipitate the cell debris. The tubes were then centrifuged and the supernatant was added to a spin column. The DNA bound to the membrane within the spin column. To remove impurities, wash buffer was added to the spin column and centrifuged. Finally, the DNA was removed from the membrane using an elution buffer and the pure DNA was collected at the bottom of the tube. This resulted in the promoterless control plasmid pEGFP-N1(CMV-). The DNA concentration was measured using the NanoDrop 2000 (Thermo Scientific).

2 Cell culture

MCF7 human breast cancer cells (ATCC), C2C12 mouse skeletal myoblast cells (ATCC), and human embryonic kidney cells (HEK-293) were cultured in growth media consisting of low-glucose Dulbecco's Modified Eagle's Medium (DMEM; Hyclone) containing 10% fetal bovine serum (FBS; Hyclone) and 1% penicillin/streptomycin (Hyclone) on polystyrene cell culture dishes (BD Sciences/Sarstedt/Grenier Bio One). Cells were incubated at 37°C and 5% CO_2 and were maintained by washing with phosphate buffered saline (PBS) and replacing growth media every other day unless otherwise specified. C2C12 differentiation was induced the day after transfection by replacing growth media with differentiation media consisting of low-glucose DMEM containing 2% horse serum (Hyclone) and 1% penicillin/streptomycin.

Human skeletal muscle myoblast cells (HSMM; Lonza) were cultured in Skeletal Muscle Growth Media- 2 (SKGM-2) with 10% fetal bovine serum (FBS), 1% L-glutamine, 0.1% human epidermal growth factor, 0.1% dexamethasone, and 0.1% Gentamicin/Amphotericin-B (SKGM-2 Bullet Kit, Lonza, Rochester, NY). In order for HSMM cells to remain adhered to the culture dish during differentiation, culture dishes were treated with Cultrex for 1 hour prior to plating the cells.

Differentiation was induced by replacing SKGM-2 with differentiation media of low-glucose DMEM containing 2% horse serum (Hyclone) and 1% penicillin/streptomycin.

3 Transfection

Cells were transfected with the specified plasmids. Transfections were performed with Lipofectamine 2000 (Invitrogen), Lipofectamine 3000 (Invitrogen), or jetPrime (Polyplus-transfection) and optimised according to the manufacturer's instructions unless otherwise specified. Cells were transfected at approximately 80% confluency and incubated with the transfection mixture for 4 to 6 hours unless otherwise specified. Following the appropriate incubation time, the mixture was replaced with growth media.

4 Luciferase assay

To quantify luciferase activity, a Synergy H1 Microplate Reader (BioTek) was used. The protocol was adapted from Siebring-van Olst et al., 2013 (104). Cells were plated in an opaque, white 96 well plate (Grenier Bio One) at a density of 20 000 cells per well and transfected the following day. 48 hours post-transfection, growth media was removed, and cells were washed three times with PBS. The final PBS wash was aspirated, and the dry plate was placed in the -80°C freezer for 10 minutes to lyse the cells. After 10 minutes, the plate was removed and allowed to return to room temperature. During this time, the appropriate volume of firefly luciferase assay reagent (FLAR; 20mM Tricine, 100µM EDTA, 1.07mM $MgCO_3$, 2.67mM $MgSO_4$, 17mM DTT, 250µM ATP, 250µM D-luciferin) was made (104). FLAR was added to each well using a multi-channel pipette. The plate was immediately placed in the plate reader and read every 3 minutes for 12 minutes. The results from the second (3 minute) reading were used for analysis.

The luciferase activity detected in the blank wells was subtracted from the rest of the data. Data was normalised to the luciferase activity of the promoterless control plasmid and expressed in relative light units (RLU).

5 Green fluorescent protein quantification

To quantify EGFP fluorescence, a Synergy H1 Microplate Reader (BioTek) was used. Cells were plated in an opaque, white 96 well plate (Grenier Bio One) at a density of 20 000 cells per well and transfected the following day. 48 hours post-transfection, cells were washed, and growth media was replaced with PBS prior to reading. The excitation and emission wavelengths of EGFP were set to 480 nm and 509 nm, respectively. Data was normalised to the fluorescence of the promoterless control plasmid and expressed as relative fluorescence units (RFU).

6 Flow cytometry

Cells were washed with warm PBS and harvested via trypsinization. Cells were centrifuged at 1000*g* for 5 minutes at 4°C, resuspended in PBS, and centrifuged again at 1000*g* for 5 minutes at 4°C. Cells were resuspended in 1 mL of PBS, counted using the Z2 Coulter Counter (Beckman-Coulter) and diluted to 1 million cells per 1 mL of PBS. GFP fluorescence was measured using flow cytometry (FACSCalibur, BD Biosciences).

7 Isolation and preparation of cell lysates

Cells in culture were washed with warm PBS and isolated via trypsinization. Collected cells were centrifuged at 1000*g* for 5 minutes at 4°C. The pellet was then re-suspended in PBS and centrifuged again at 1000*g* for 5 minutes at 4°C. To produce whole cell lysates, lysis buffer (20mM HEPES, 10mM NaCl, 1.5mM MgCl, 1mM DTT, 20% glycerol and 0.1% Triton X-100; pH 7.4) and protease inhibitors (Complete Cocktail; Roche) were added to the collected cells and

subsequently sonicated. The cell lysate protein content was determined using the BCA protein assay method.

8 Immunoblotting

Equal amounts of protein were loaded into 10-12% SDS-PAGE gels, separated by electrophoresis, and transferred onto PVDF membranes (Bio-Rad Laboratories). Membranes were blocked for 1 hour at room temperature with 5% milk-Tris-buffered saline Tween 20. Next, membranes were incubated either overnight at 4°C or for 1 hour at room temperature with primary antibodies against the following proteins: ARC (Santa Cruz Biotechnologies) and GFP (Santa Cruz Biotechnologies). Membranes were then washed with tris-buffered saline with Tween 20 (TBS-T) and incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. Protein bands were visualised using enhanced chemiluminescence western blotting detection reagents (GE Healthcare) and the ChemiGenius 2 Bio-Imaging System (Syngene).

9 Statistical analyses

All results are presented as means ± standard error of the mean. Comparisons were analysed using one-way analyses of variance (ANOVA) with Tukey's post-hoc comparisons, or unpaired T-tests where appropriate. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using Microsoft Excel and GraphPad Prism 6.

Results

1 Modification of the pGL3-0.75ARC-GFP(luc-) plasmid

As C2C12 cells differentiate, ARC protein levels increase (Figure 8). Therefore, C2C12 cell differentiation was used to show the relationship between ARC protein levels and GFP protein levels in cells transfected with a GFP reporter plasmid containing part of the ARC promoter region. Theoretically, as transfected C2C12 cells differentiate and ARC protein levels increase, GFP fluorescence should also increase.

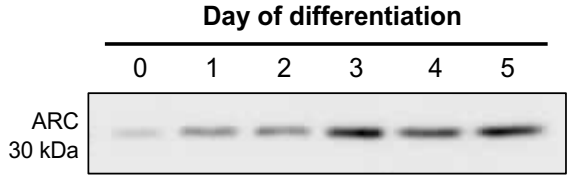


Figure 10: As C2C12 cells differentiate, ARC protein levels increase. Representative immunoblot of whole cell lysate protein levels of ARC.

A pGL3-basic plasmid containing 0.75 kB of the human ARC promoter region most proximal to the translational start site of the short transcript variants (pGL3-0.75ARC; Figure 9) created by Dr. Roger Foo (National University of Singapore) was modified to encode green fluorescent protein (GFP) by Darin Bloemberg. This resulted in a pGL3-0.75ARC-GFP(luc-) plasmid (hereinafter referred to as FOO-ARC-GFP). When the FOO-ARC-GFP plasmid was transfected into C2C12 cells, it was evident that as ARC protein levels increased, the GFP fluorescence decreased. Further analysis of the sequencing showed that a stop codon was present and in-frame just before the EGFP sequence, resulting in the loss of GFP protein production when the ARC promoter region was activated. The following primers were used to modify this region of the plasmid: forward, 5'-TAC GCG TGC TAG CCC GGG CTC GAG-3'; reverse, 5'-AAG CTT CCG CTC CTG CGC GTT GCC CAT-3'. Following amplification, the modified sequence was ligated into the pGL3-GFP(luc-) plasmid. The resulting plasmid

((pGL3-0.75MODARC-GFP(luc-)) and the pGL3-GFP(luc-) promoterless control plasmid were verified by DNA sequencing (performed by The Centre for Applied Genomics, The Hospital for Sick Children) (Appendix Figure 1 and 2).

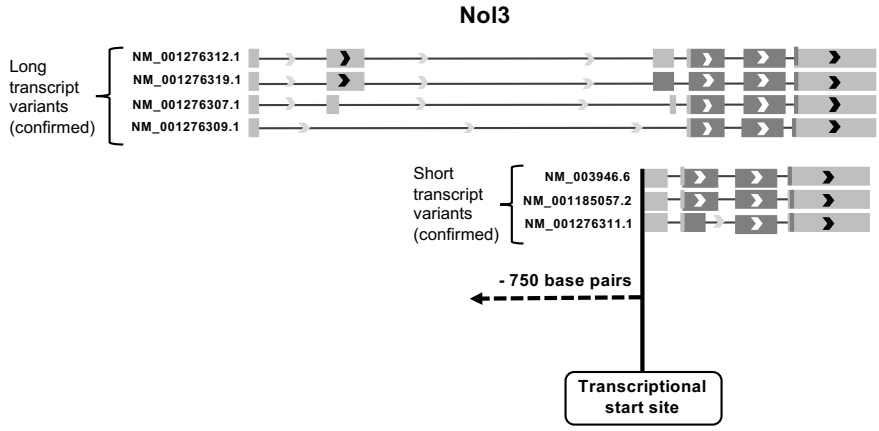


Figure 11: The ARC promoter region included in the pGL3-0.75ARC plasmid is in relation to the transcriptional start site of the short transcript variants. Adapted from NCBI Gene Report (Gene ID 8996).

2 Transfections with the pGL3-0.75MODARC(luc-) plasmid

Similar to the previous experiment, C2C12 cells were transfected with the pGL3-0.75MODARC-GFP(luc-) plasmid (hereinafter referred to as FOO-ModARC-GFP) or a pGL3-GFP(luc-) promoterless control plasmid (hereinafter referred to as the promoterless control) using a ratio of 1 μg DNA to 3 μL of Lipofectamine2000 reagent and differentiation was induced the next day. 24 hours after differentiation was induced, limited GFP fluorescence was detected under the fluorescent microscope (Figure 10). The following day, there was no GFP fluorescence detectable under the fluorescent microscope.

This outcome suggested that there were still some underlying issues with the design of the plasmid or the cell model used. Therefore, the next step was to use a different cell line that was of human origin and displayed basal ARC protein, as opposed to C2C12 cells that induce ARC protein as they differentiate. Consequently, high pass MCF7 cells (HP MCF7 cells) were used. While highly passaged cells typically do not retain the same qualities as low passage cells (105),

HP MCF7 cells still contain a high ARC protein content (Figure 11) and are therefore a viable model to show the potential relationship between ARC protein levels and GFP protein levels. HP MCF7 cells were transfected with the FOO-ModARC-GFP plasmid and the promoterless control plasmid using a ratio of 2 μg DNA to 6 μL of Lipofectamine2000 reagent. 24 and 48 hours post-transfection, no GFP fluorescence was detected under the fluorescent microscope.

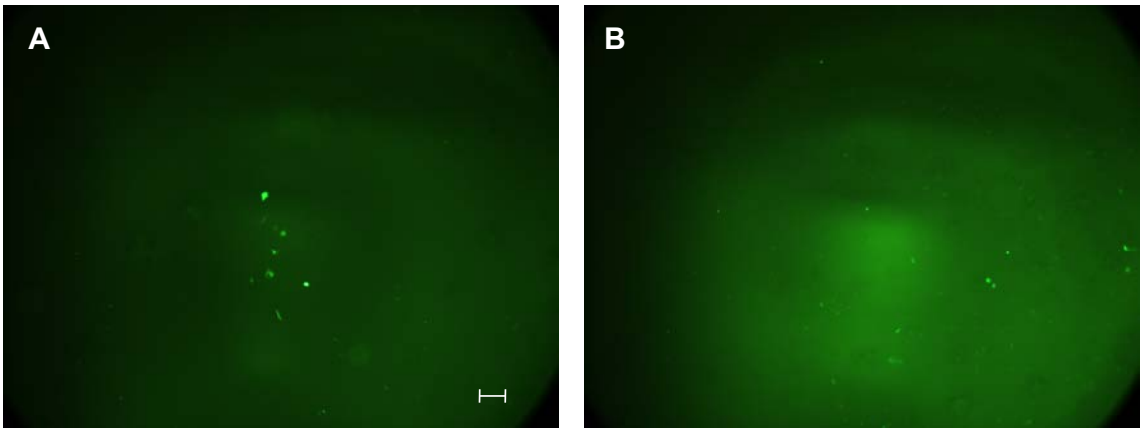


Figure 12: C2C12 cells were transfected and differentiation was induced the following day. On Day 1 of differentiation, limited GFP fluorescence was detected. A) C2C12 cells transfected with the pGL3-0.75MODARC-GFP(luc-) plasmid. B) C2C12 cells transfected with the pGL3-GFP(luc-) promoterless control. Bar represents 200 μm.

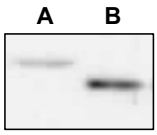


Figure 13: While HP MCF7 cells are physiologically and morphologically different than LP MCF7 cells, ARC protein is still highly expressed. Representative immunoblot of whole cell lysate protein levels of ARC. A: C2C12 cell standard, B: HP MCF7 cells.

To rule out that this result was not due to a poor transfection efficiency, a different transfection reagent and protocol was used. HP MCF7 cells were transfected with the FOO-ModARC-GFP plasmid and the promoterless control plasmid using a ratio of 1 μg DNA to 2 μL of jetPrime reagent. 24 and 48 hours post-transfection, no GFP fluorescence was detected under the fluorescent microscope. To be certain there was not a problem with the transfection protocols, HP MCF7 cells were transfected with a pDsRed2-Mito plasmid, an XIAP-GFP plasmid

and the FOO-ModARC-GFP plasmid using either the Lipofectamine2000 reagent or the jetPrime reagent at various DNA to reagent ratios. The following day, DsRed and GFP fluorescence was detected in the cells transfected with the pDsRed2-Mito and XIAP-GFP plasmids; however, no GFP fluorescence was detected in any cells transfected with the FOO-ModARC-GFP plasmid. These results showed that the transfection protocols were successful in transporting plasmid DNA into the cells.

Since there was no problem with the transfection efficiency, HP MCF7 cells were once again transfected with the FOO-ModARC-GFP plasmid and the promoterless control plasmid using a ratio of 1 μg DNA to 2 μL of jetPrime reagent for a more thorough analysis of the fluorescence. The following day, no GFP fluorescence was detected using the fluorescent microscope. 48 hours post-transfection, live cells were collected, and GFP fluorescence was analysed using an end-point GFP fluorescence assay (Figure 12). Cells were added to the wells of a black 96 well plate (Grenier Bio One) at different concentrations in triplicate. GFP fluorescence was not higher in cells transfected with the FOO-ModARC-GFP plasmid compared to cells transfected with the promoterless control plasmid when less than 50 000 cells were added to each well. However, when 50 000 cells were added to each well, GFP fluorescence was slightly higher in cells transfected with the FOO-ModARC-GFP plasmid compared to cells transfected with the promoterless control plasmid. This trend continued when 100 000 cells were added to each well. Based on this result, it is possible that the plate reader was not sensitive enough to detect changes in GFP fluorescence when there were less than 50 000 cells added to each well. Nevertheless, the differences detected when 50 000 and 100 000 cells were added to each well were very small. Therefore, these cells were also analysed using flow cytometry. In cells transfected with the FOO-ModARC-GFP plasmid, flow cytometry analysis revealed that only 3.64% of the cells in the sample were GFP

positive (Figure 13). The flow cytometry results, in combination with the plate reader analysis, made it clear that the plasmid was not producing a significant amount of GFP protein with ARC promoter activation.

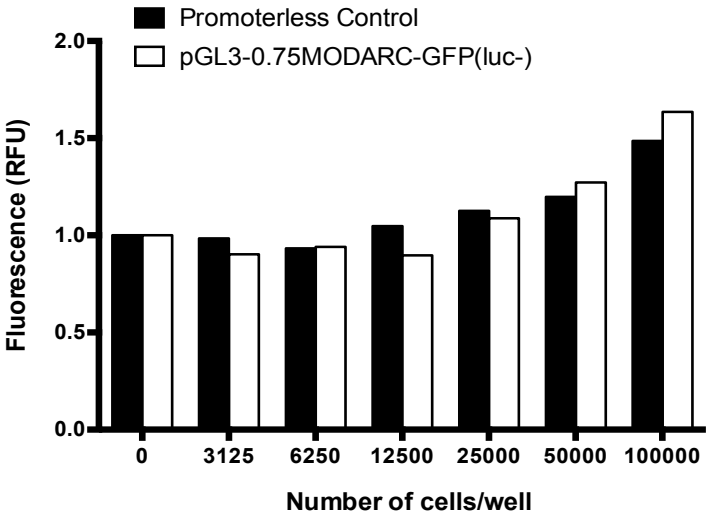


Figure 14: When 50 000 or 100 000 cells were added to each well of a 96 well plate, GFP fluorescence was slightly higher in cells transfected with the FOO-ModARC-GFP plasmid compared to the promoterless control. GFP fluorescence was analysed using an end-point GFP fluorescence plate reader assay (n=1, in triplicate).

Empty GFP

Histogram Statistics

File: GFP MCF7.001 Acquisition Date: 19-Jun-15
Gate: G1 Gated Events: 7494
Total Events: 7920 X Parameter: FL1-H (Log)

Marker	Events	% Gated	% Total	Mean	Geo Mean
All	7494	100.00	94.62	3.56	3.26
NO GFP	7383	98.52	93.22	3.42	3.19
GFP	114	1.52	1.44	12.99	12.56

ARC 750 GFP

Histogram Statistics

File: GFP MCF7.002 Acquisition Date: 19-Jun-15
Gate: G1 Gated Events: 6843
Total Events: 7440 X Parameter: FL1-H (Log)

Marker	Events	% Gated	% Total	Mean	Geo Mean
All	6843	100.00	91.98	3.74	3.30
NO GFP	6601	96.46	88.72	3.40	3.14
GFP	249	3.64	3.35	12.91	12.39

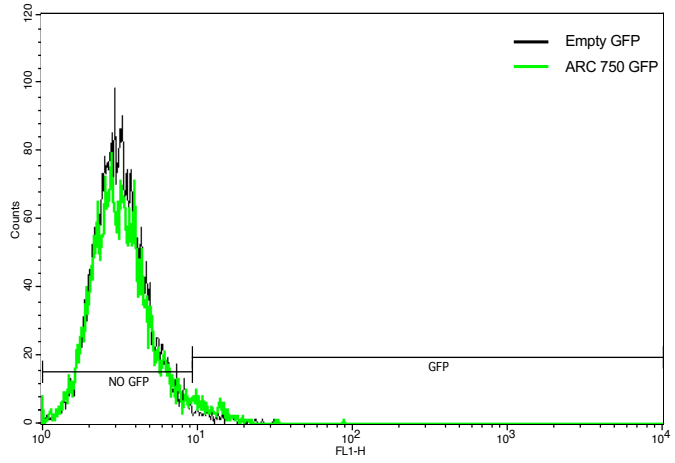


Figure 15: In HP MCF7 cells transfected with the FOO-ModARC-GFP plasmid, only 3.64% of cells were GFP positive. **"**ARC 750 GFP" represents cells transfected with the pGL3-0.75MODARC-GFP(luc-) plasmid and "Empty GFP" represents cells transfected with the promoterless control plasmid.

Next, HP MCF7 cells were transfected with the FOO-ModARC-GFP plasmid and starved in an attempt to manipulate ARC protein levels. Starvation increases the transcription factor Foxo3 in C2C12 cells (106). In a study by Lu and colleagues, it was found that the transcription factor Foxo3a bound to and activated the rat ARC promoter region, which resulted in an increase in ARC protein content (107). Therefore, cells were incubated overnight in Hank's Balanced Salt Solution or DMEM free of FBS. Unfortunately, starvation did not result in detectable GFP fluorescence under the fluorescent microscope the following day. It is important to note that this may have been because starvation did not increase ARC protein levels in MCF7 cells, as there are physiological differences between rodent and human cell lines; however, it may also indicate that the FOO-ModARC-GFP plasmid was not responding to ARC promoter activation.

To determine if the FOO-ModARC-GFP plasmid would respond to ARC induction, the skeletal muscle differentiation model was used again. Since the ARC promoter region in the FOO-ModARC-GFP plasmid is human, human skeletal muscle myoblasts (HSMM) were transfected with the FOO-ModARC-GFP plasmid and the promoterless control plasmid using a ratio of 1 μg DNA to 3 μL of Lipofectamine2000 reagent or 2 μg DNA to 6 μL of Lipofectamine2000 reagent. Previous work in the lab has shown that, similar to C2C12 cells, ARC expression in HSMM cells increases during differentiation. After incubation with the FOO-ModARC-GFP plasmid DNA and reagent, cells did not appear to be healthy. Cells were allowed to recover in growth media for two days, then differentiation was induced. No GFP fluorescence was detected under the fluorescent microscope throughout the differentiation period.

HP MCF7 cells were once again transfected with the FOO-ModARC-GFP plasmid and the promoterless control plasmid using a ratio of 2 μg DNA to 6 μL of Lipofectamine2000 reagent with the intension of being collected for ARC and GFP immunoblot analysis. This time, these cells

were transfected at a lower confluency than on previous occasions (approximately 60%). Surprisingly, GFP fluorescence was detected under the fluorescent microscope the following day (Figure 14A). 48 hours post-transfection, GFP fluorescence could still be detected (Figure 14B). This experiment was repeated to confirm the results (Figure 14C and 14D). Interestingly, there was no difference in the protocol used in this experiment other than the confluency at which the cells were transfected.

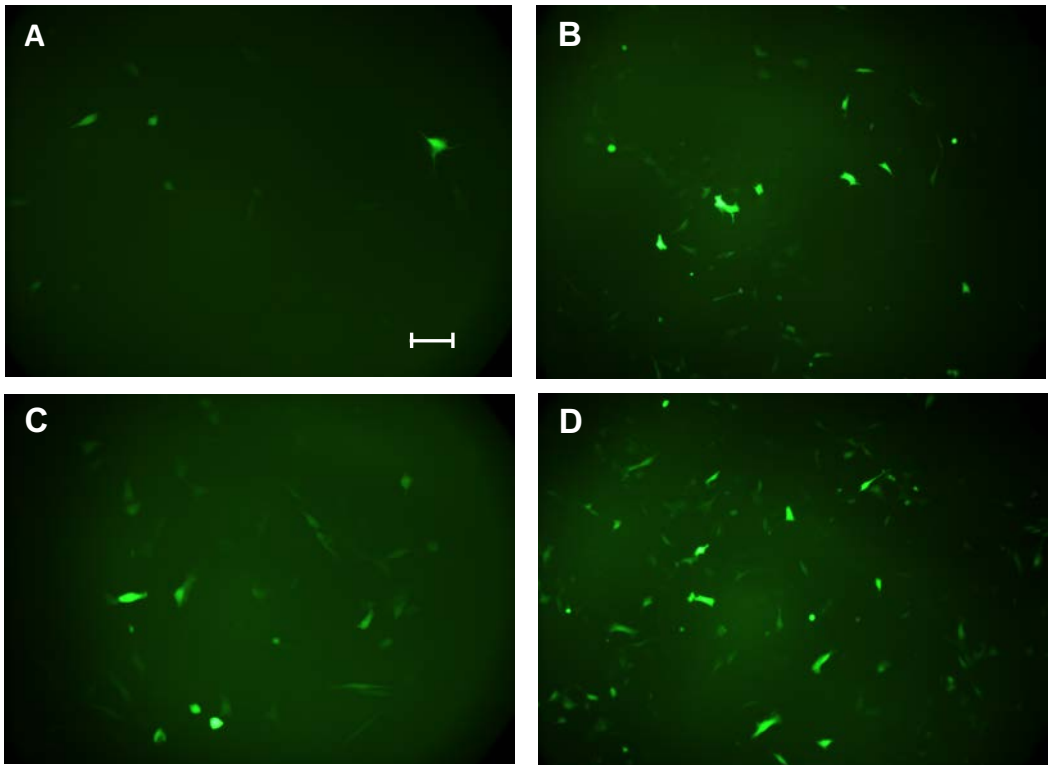


Figure 16: GFP fluorescence was detected under the fluorescent microscope in transfected HP MCF7 cells. A) 24 hours post-transfection. B) 48 hours post-transfection. The experiment was repeated to confirm the results. C) 24 hours post-transfection. D) 48 hours post-transfection. Bar represents 100 μm.

Given this promising result, C2C12 cells were transfected at a lower confluency with the FOO-ModARC-GFP plasmid and the promoterless control plasmid using a ratio of 2 μg DNA to 6 μL of Lipofectamine2000 reagent. Given that the previous experiment established that the FOO-ModARC-GFP plasmid produced GFP in a cell line with basal levels of ARC protein, the

purpose of this experiment was to determine whether GFP protein levels would increase alongside the increase in ARC protein levels that occurs with skeletal muscle differentiation. The following day, differentiation was induced. Cells were collected over the differentiation period and analysed via immunoblotting. ARC protein content increased as the cells differentiated and GFP protein was detected on Day 0 and Day 1, but not after Day 2 in cells transfected with the FOO-ModARC-GFP plasmid (Figure 15). While this was an improvement on the previous C2C12 cell experiments, GFP protein levels did not follow the same trend as ARC protein levels in C2C12 differentiation. It is important to note that the C2C12 experiments may not be effective because there are differences in the ARC promoter region between mouse and human; however, human skeletal muscle myoblasts were not used at this time because of their inability to handle the transfection protocol in the previous experiment.

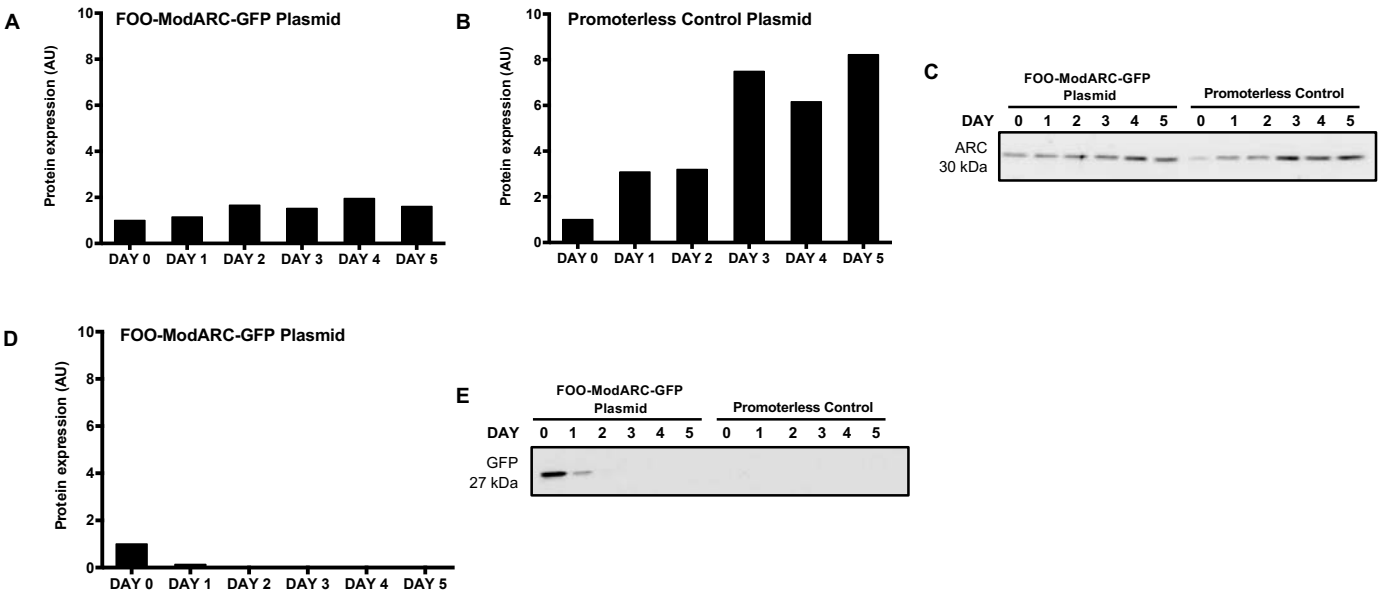


Figure 17: While ARC protein content increased as C2C12 cells differentiated, GFP protein content did not follow the same trend. A) Quantification of ARC protein levels in C2C12 cells transfected with the FOO-ModARC-GFP plasmid. B) Quantification of ARC protein levels in C2C12 cells transfected with the promoterless control plasmid. C) Whole cell lysate immunoblot of ARC. D) Quantification of GFP protein levels in cells transfected with the FOO-ModARC-GFP plasmid. E) Whole cell lysate immunoblot of GFP. Day 0 – Day 5 represent day of differentiation. (n=1)

Because the previous experiments were done on HP MCF7 cells, low pass MCF7 (LP MCF7) cells were cultured and transfected to determine if a similar ARC and GFP protein level relationship could be seen. LP MCF7 cells were transfected with the FOO-ModARC-GFP plasmid using a ratio of 1 μg DNA to 3 μL of Lipofectamine2000 reagent or 2 μg DNA to 6 μL of Lipofectamine2000 reagent. The following day, the cells were dead. New transfection protocols were found in an attempt to limit the number of dead cells in the next experiment. LP MCF7 cells were transfected with the FOO-ModARC-GFP plasmid using the Maartje Vogel MCF7 Lipofectamine2000 transfection protocol (Appendix Figure 3) or a ratio of 1 μg DNA to 2 μL of jetPrime reagent. The following day, no GFP fluorescence was detected using the fluorescent microscope. All the cells were dead 48 hours post-transfection. Next, the Lipofectamine2000 protocol from Life Technologies (Appendix Figure 4) was followed with no modifications and there was no GFP fluorescence detected 24 hours post-transfection. Cells were dead the following day. There was no explanation as to why the LP MCF7 cells were dying with transfection, thus other cells types were used.

Experiments in the literature using the original pGL3-0.75ARC plasmid from Dr. Roger Foo used HEK-293 cells and therefore, the same cells were used in this experiment (75). HEK-293 cells are known to express very low endogenous levels of ARC protein (61). However, it was discovered that ARC protein levels increased as the cells became more confluent. Thus, these cells were used as an alternative method to show the relationship between ARC protein levels and GFP protein production. HEK-293 cells were transfected with the FOO-ModARC-GFP plasmid using a ratio of 2 μg DNA to 6 μL of Lipofectamine2000 reagent and allowed to grow for six days. GFP fluorescence was not detected under the fluorescent microscope on day 1, but was detectable on day 2 and 3. After this time, GFP fluorescence began to decrease (Figure 16A). Cells were

collected each day and analysed via immunoblotting (Figure 16B-E). While there were no significant differences in ARC protein content across the days in culture, it is clear that ARC protein content increased the longer the cells were in culture. GFP protein content was detectable on day two, but progressively decreased until day four. After day four, no GFP protein was detected. GFP fluorescence detected under the fluorescent microscope appeared to follow a similar trend as that seen in the immunoblot. However, the GFP fluorescence and protein levels did not increase as ARC protein levels increased.

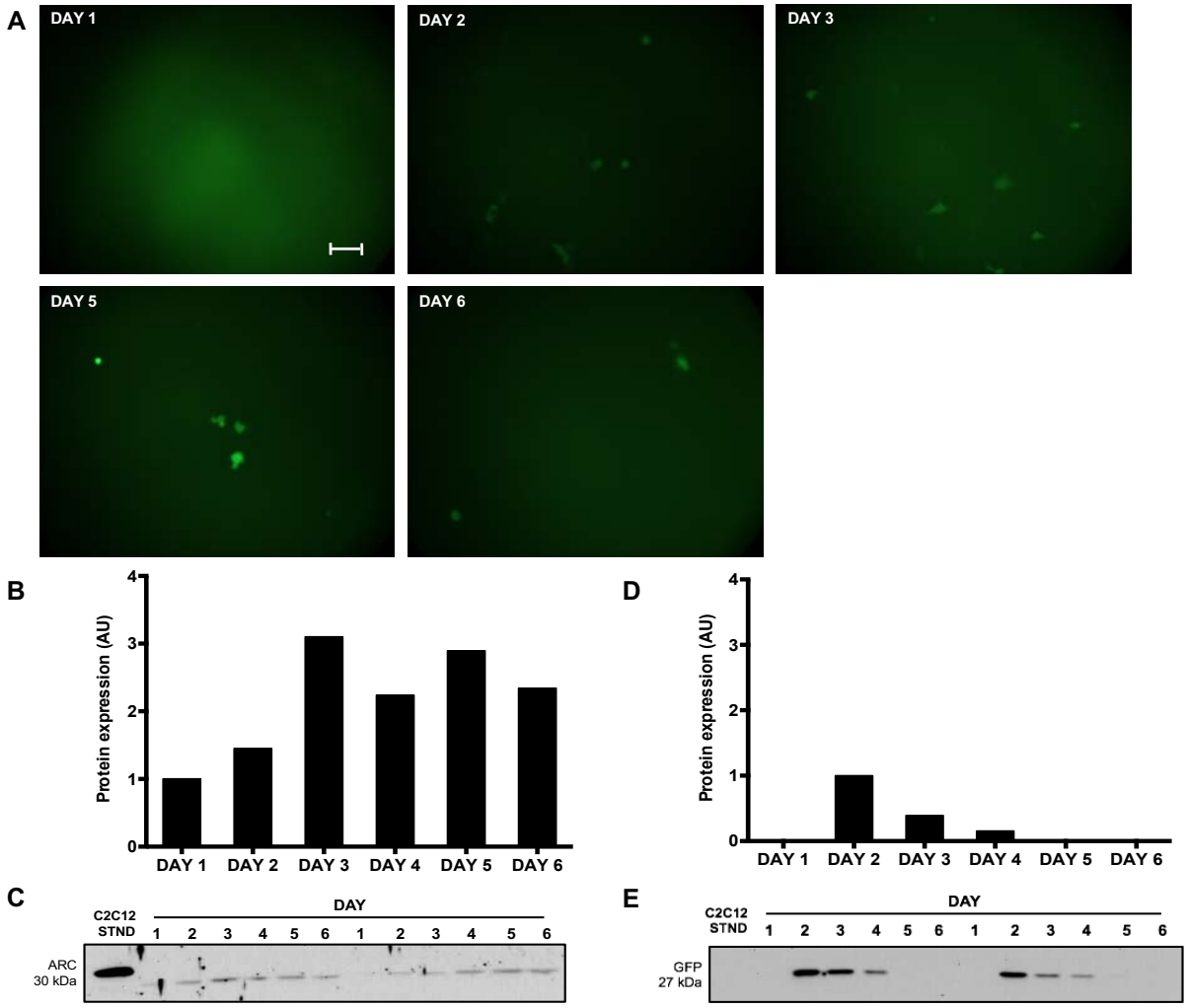
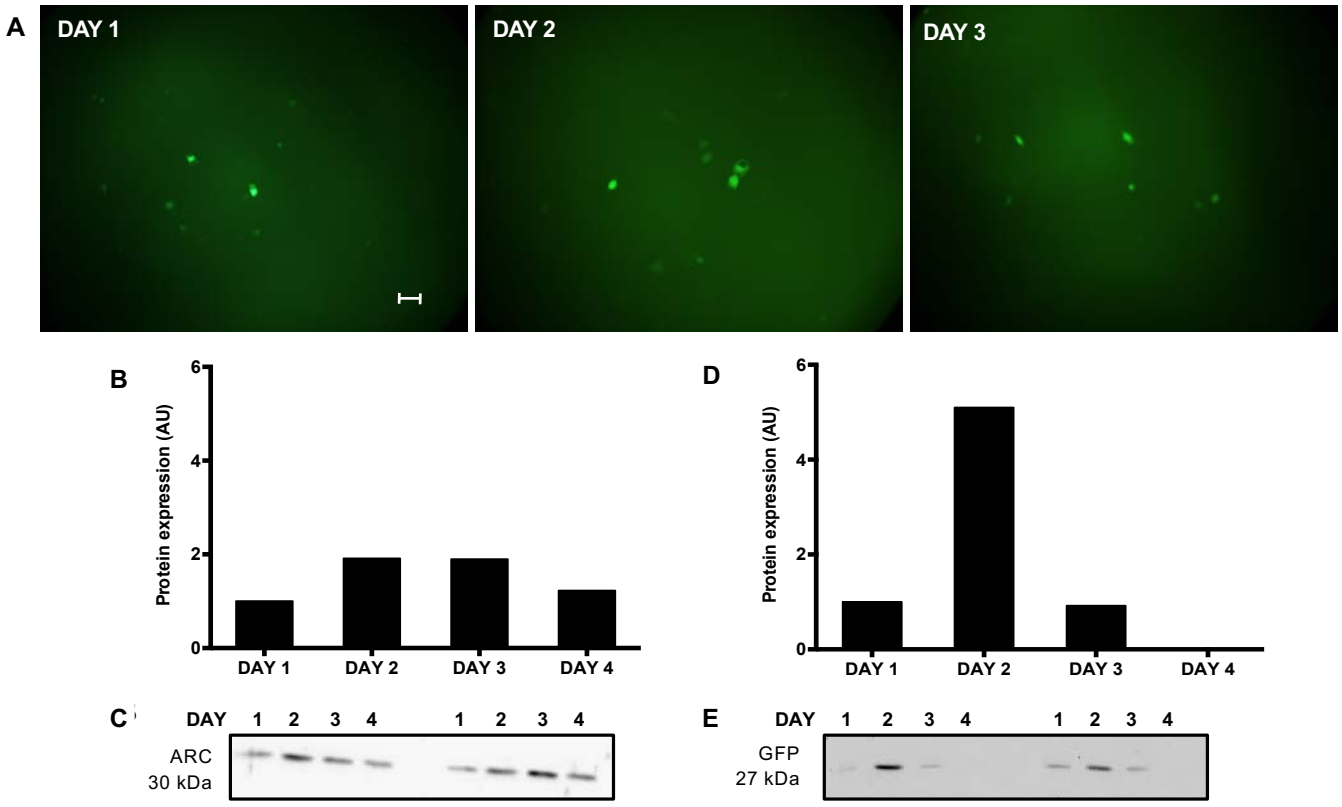


Figure 18: ARC protein levels slightly increases as HEK-293 cells become more confluent. However, GFP protein levels did not follow the same trend as ARC protein levels in HEK-293 cells. A) GFP fluorescence was detected under the fluorescent microscope in transfected HEK-293 cells. However, it was expected for the fluorescence to increase as the cells became more confluent (NOTE: An image of GFP fluorescence detected on day 4 was not taken). Bar represents 100 μm. B) Quantification of ARC protein levels. C) Whole cell lysate immunoblot of ARC. D) Quantification of GFP protein levels. E) Whole cell lysate immunoblot of GFP. (n=1, in duplicate).

Next, a new transfection reagent, Lipofectamine3000, was used in LP MCF7 cells. Lipofectamine3000 results in a 2-fold improvement in expression over Lipofectamine2000 in MCF7 cells when transfecting with plasmid DNA (108). Therefore, LP MCF7 cells were transfected with the FOO-ModARC-GFP plasmid using a ratio of 1 μg DNA to 6 μL of Lipofectamine3000 reagent with the addition of 3.75 μL of the P3000 reagent. The following day, limited GFP fluorescence was detected under the fluorescent microscope (Figure 17A). Next, LP MCF7 cells transfected using 3.75 μL of the P3000 reagent were cultured for four days, then collected and analysed via immunoblotting (Figure 17B-E). While ARC protein content was high across all four days, GFP protein content peaked on day two, then decreased. In this case, it was clear that the Lipofectamine3000 reagent did not improve the GFP protein levels over the other reagents used in the previous experiments.



(Previous page) Figure 19: GFP protein was not highly expressed in LP MCF7 cells transfected with the FOO-ModARC-GFP plasmid using Lipofectamine3000 with the addition of 3.75µL of P3000 reagent. A) Some GFP fluorescence was detected under the fluorescent microscope in transfected LP MCF7 cells. Bar represents 100 µm. B) Quantification of ARC protein levels. C) Whole cell lysate immunoblot of ARC. D) Quantification of GFP protein levels. E) Whole cell lysate immunoblot of GFP. (n=1, in duplicate)

3 Luciferase assay

In light of the results of the prior experiments, the original pGL3-0.75ARC plasmid without any modifications was analysed using a luciferase assay. A pGL3-basic plasmid containing 1.005 kB of the human ARC gene (pGL3-1.005ARC) that was obtained from Dr. Olga Razorenova (University of California, Irvine) and a promoterless pGL3-basic control plasmid were also analysed. Each plasmid was transfected into LP MCF7 cells using a ratio of 1 µg DNA to 2 µL of jetPrime reagent, and luciferase activity was analysed 48 hours later. The amount of luciferase activity from the pGL3-0.75ARC plasmid was similar to that of the promoterless pGL3-basic control, while the pGL3-1.005ARC plasmid had significantly more activity than both the promoterless control and the pGL3-0.75ARC plasmid ($p<0.05$) (Figure 18). It was evident that the pGL3-0.75ARC plasmid was not producing the reporter protein in response to ARC promoter activation even in its original state.

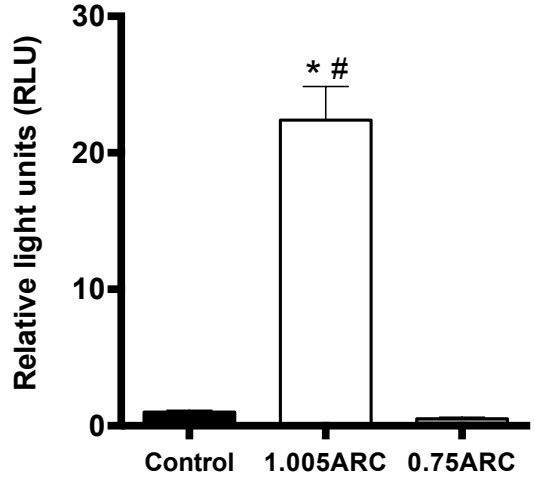


Figure 20: Luciferase activity of the promoterless control plasmid, the pGL3-1.005ARC plasmid, and the pGL3-0.75ARC plasmid. The pGL3-0.75ARC plasmid had less luciferase activity than expected. * $p < 0.05$ compared to the control, # $p < 0.05$ compared to 0.75ARC (n=3, in triplicate).

4 Modification of the pGL3-1.005ARC plasmid

Given the results of the luciferase assay, the pGL3-basic plasmid containing 1.005 kB of the human ARC gene was modified to encode EGFP. This 1.005 kB region contains 701 base pairs of the human ARC promoter most proximal to the transcriptional start site of the long transcript variant, the first exon, and part of the first intron (Figure 19). The coding sequence for luciferase was excised via a HindIII and XbaI digest. The same EGFP segment used in the modification of the pGL3-0.75ARC plasmid was ligated into the pGL3-1.005ARC(luc-) plasmid. The resulting plasmid (pGL3-1.005ARC-GFP(luc-)) was verified by DNA sequencing (performed by The Centre for Applied Genomics, The Hospital for Sick Children) (Appendix Figure 5).

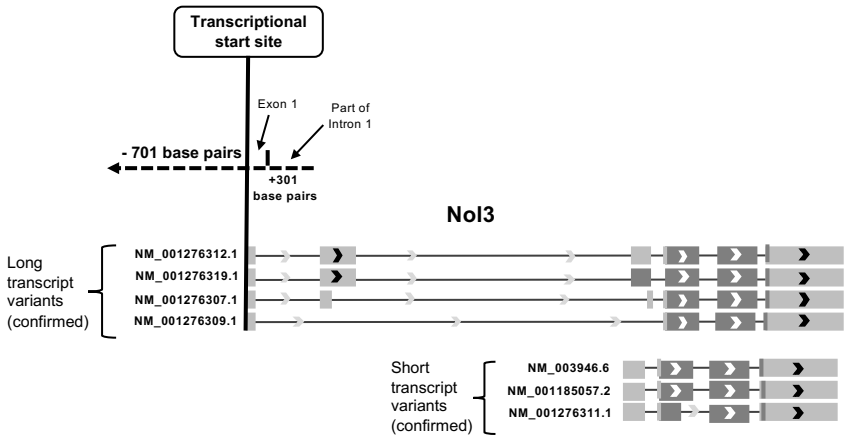


Figure 21: The ARC promoter region included in the pGL3-1.005ARC plasmid is in relation to the transcriptional start site of the long transcript variants. Adapted from NCBI Gene Report (Gene ID 8996).

5 Transfections with the pGL3-1.005ARC-GFP(luc-) plasmid

As mentioned previously, ARC protein levels increased as HEK-293 cells became more confluent. Therefore, HEK-293 cells were transfected with the pGL3-1.005ARC-GFP(luc-) plasmid (hereinafter referred to as RAZ-ARC-GFP(luc-)) and a pDsRed2-Mito plasmid using a ratio of 1 μg DNA to 2 μL of jetPrime reagent. The transfection mixture was removed after 5 hours. HEK-293 cells were plated at various concentrations that would result in the cells being at various confluencies at the time of transfection, and therefore have different levels of ARC protein.

The following day, DsRed fluorescence was detected in cells transfected with the pDsRed2-Mito plasmid, but no GFP fluorescence was detected under the fluorescent microscope in cells transfected with the RAZ-ARC-GFP(luc-) plasmid.

Immunoblot analysis revealed that ARC protein was not as highly expressed in confluent HEK-293 cells in comparison to other cell types, including MCF7 cells (Figure 20). Therefore, it was possible that the amount of ARC expression in confluent HEK-293 cells may have been too low to result in a detectable amount of GFP fluorescence under the fluorescent microscope. Therefore, LP MCF7 cells, which contain the highest ARC protein content out of the cell lines tested, were transfected with the RAZ-ARC-GFP(luc-) plasmid using various DNA and reagent concentrations. However, the following day, no GFP fluorescence was detected in any cells under the fluorescent microscope.

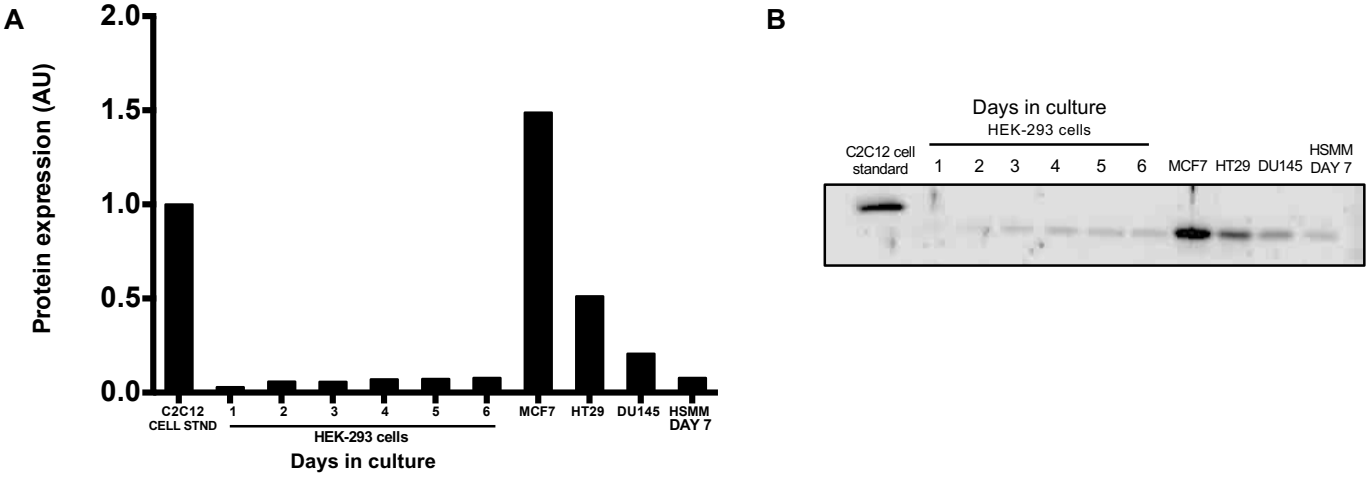


Figure 22: ARC protein levels in different cell lines. ARC protein content in HEK-293 cells is much lower than in MCF7 and C2C12 cells. A) Quantification of ARC protein levels. B) Whole cell lysate immunoblot of ARC (n=1).

6 Modification of the pGL3-1.005ARC-GFP(luc-) plasmid

Given the results of the previous experiments, the RAZ-ARC-GFP(luc-) plasmid was modified. Rather than replacing the luciferase segment in the pGL3-1.005ARC plasmid with the

EGFP segment from the pEGFP-N1 plasmid, the 1.005 kB segment of the ARC gene was cut out of the original pGL3-1.005ARC plasmid and ligated into the pEGFP-N1 plasmid. The CMV promoter present in the pEGFP-N1 vector was removed via an AseI and BamHI digest. The following primers were used to add an AseI restriction site at the start and a BglII restriction site at the end of the 1.005 kB segment of the ARC gene in the original plasmid from Dr. Razorenova: forward, 5'-TAA GCA ATT AAT AAG GGG CTT GGA ACC AGT CC-3'; reverse, 5'-TGC TTA AGA TCT GTG CGA CTG CAC GGA TTT TC-3'. Following amplification, the 1.005 kB segment of the ARC gene was ligated into the pEGFP-N1(CMV-) plasmid. The resulting plasmid (pEGFP-N1-1.005ARC(CMV-)) was verified by DNA sequencing (performed by The Centre for Applied Genomics, The Hospital for Sick Children) (Appendix Figure 6).

7 Transfections with the pEGFP-N1-1.005ARC(CMV-) plasmid

First, LP MCF7 cells were transfected with the pEGFP-N1-1.005ARC(CMV-) plasmid (hereinafter referred to as RAZ-ARC-EGFP) and a pEGFP-N1 positive control plasmid using a ratio of 1 μg DNA to 2 μL of jetPrime reagent. 24 and 48 hours post-transfection, there was a limited amount of GFP fluorescence detected under the fluorescent microscope in cells transfected with the RAZ-ARC-EGFP plasmid, but GFP fluorescence was detected in cells transfected with the pEGFP-N1 positive control plasmid (Figure 21). Once again, it was clear that the transfection of the MCF7 cells was successful given that there was GFP fluorescence in the cells transfected with the pEGFP-N1 positive control plasmid. However, only a small amount of GFP was detected in cells transfected with the RAZ-ARC-EGFP plasmid, even though MCF7 cells have a high ARC protein content.

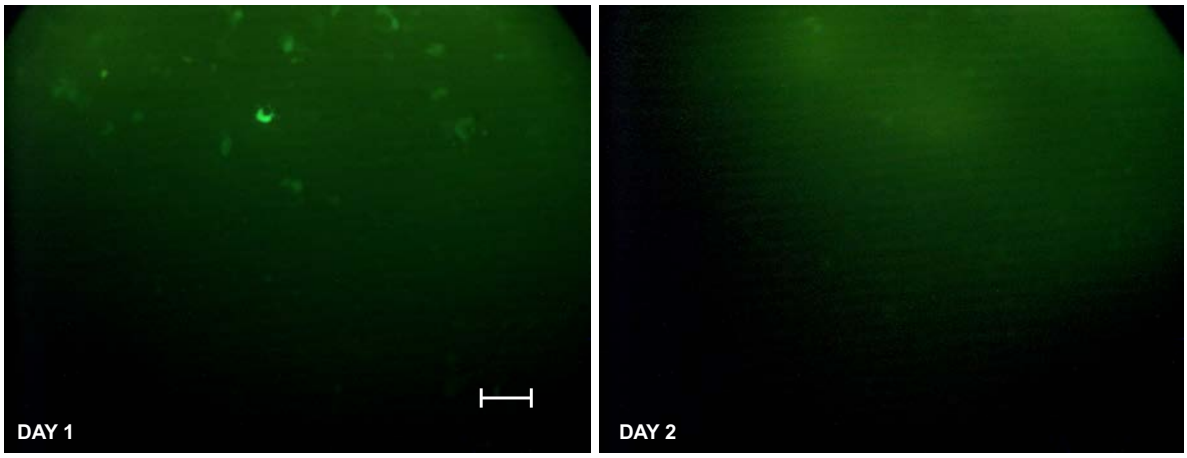


Figure 23: Limited GFP fluorescence was detected under the fluorescent microscope 24 and 48 hours post-transfection in LP MCF7 cells transfected with the RAZ-ARC-EGFP plasmid. Bar represents 100 μm.

8 Inducing hypoxia inducing factor 1 alpha as a means of increasing ARC expression

The human 1.005 kB segment of the human ARC gene used in the plasmid contains hypoxia response elements (HRE), which are binding sites for hypoxia inducing factor 1 alpha (HIF-1α) (80). When HIF-1α binds to the HRE2 site located in the ARC promoter region, ARC expression increases (80,109). Therefore, in an attempt to activate the ARC promoter and hopefully GFP protein production, HIF-1α expression was induced using cobalt chloride ($CoCl_2$) (110). LP MCF7 cells were treated with 0 μM, 75 μM, 100 μM or 125 μM $CoCl_2$ made up in growth media for 0, 12, 18, or 24 hours. It appeared that 125 μM $CoCl_2$ increased ARC expression the most after a 24 hour incubation period compared to the untreated, 0 hour control cells (Figure 22).

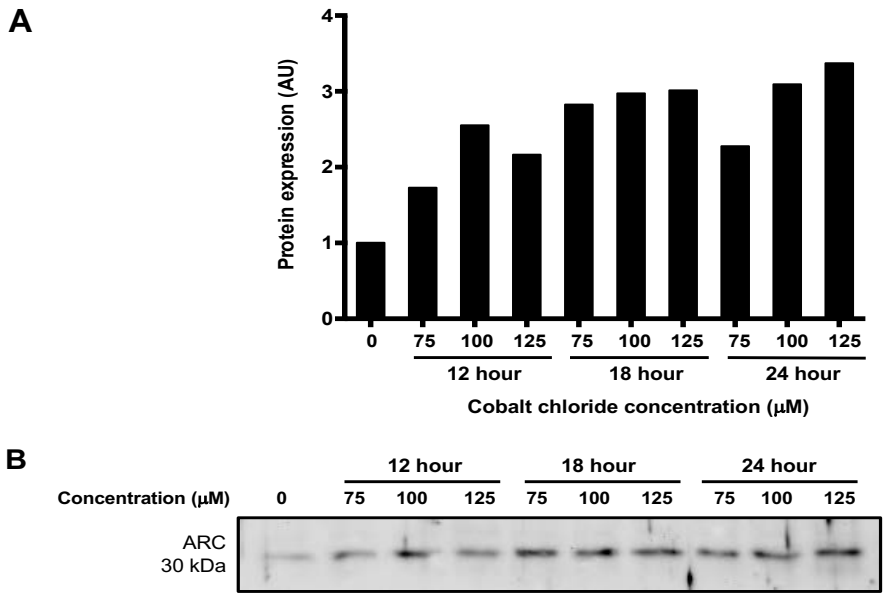


Figure 24: ARC protein levels in LP MCF7 cells when incubated in various concentrations of cobalt chloride for 0, 12, 18, or 24 hours. A) Quantification of ARC protein levels. B) Whole cell lysate immunoblot of ARC (n=1).

Next, LP MCF7 cells were transfected with the appropriate plasmids and treated with $CoCl_2$. LP MCF7 cells were transfected with the RAZ-ARC-EGFP plasmid, the pEGFP-N1 positive control plasmid and the pEGFP-N1(CMV-) negative control plasmid using a ratio of 1 μg DNA to 2 μL of jetPrime reagent. The following day, no GFP fluorescence was detected under the fluorescent microscope in cells transfected with the pEGFP-N1-1.005ARC(CMV-) plasmid. 48 hours post-transfection, cells were treated with 125 μM $CoCl_2$ for 24 hours, but no GFP fluorescence was detected under the fluorescent microscope following the treatment period in cells transfected with the RAZ-ARC-EGFP plasmid.

LP MCF7 cells were again transfected with the RAZ-ARC-EGFP plasmid, the pEGFP-N1 positive control plasmid and the pEGFP-N1(CMV-) negative control plasmid using a ratio of 1 μg DNA to 2 μL of jetPrime reagent. 48 hours post-transfection, cells were treated with 125 μM $CoCl_2$ for 24 hours. After the 24 hour incubation period, plate reader analysis showed there was no

significant difference in GFP fluorescence with $CoCl_2$ treatment in cells transfected with the RAZ-ARC-EGFP plasmid (Figure 23).

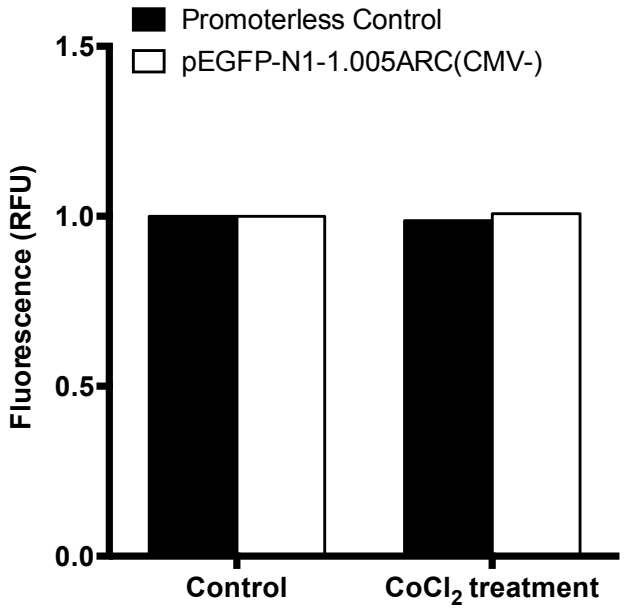


Figure 25: There was no difference in GFP fluorescence between cells transfected with the pEGFP-N1(CMV-) promoterless control plasmid and the RAZ-ARC-EGFP plasmid when treated with $CoCl_2$. "Control" represents cells that were not treated. Cells were treated with 125 μM $CoCl_2$ for 24 hours (n=1, in hextuplicate).

9 C2C12 cell transfection and differentiation

C2C12 cell differentiation was once again used as a means of increasing ARC expression in order determine if the RAZ-ARC-EGFP plasmid would respond to increasing ARC expression by progressively increasing GFP expression across the C2C12 cell differentiation period. C2C12 cells were transfected with the RAZ-ARC-EGFP plasmid, the pEGFP-N1 positive control plasmid and the pEGFP-N1(CMV-) negative control plasmid using a ratio of 1 μg DNA to 2 μL of jetPrime reagent. The following day, differentiation was induced. Surprisingly, GFP fluorescence was detectable under the fluorescent microscope in cells transfected with the RAZ-ARC-EGFP plasmid throughout the differentiation process (Figure 24A). Plate reader analysis showed that GFP fluorescence in cells transfected with RAZ-ARC-EGFP plasmid was significantly higher than in cells transfected with the pEGFP-N1(CMV-) negative control plasmid ($p<0.05$) (Figure 24B). However, there was not a significant difference in GFP fluorescence throughout differentiation in

cells transfected with the RAZ-ARC-EGFP plasmid (Figure 24C). Interestingly, immunoblot analysis revealed a pronounced increase in GFP protein content as cells differentiated (Figure 25).

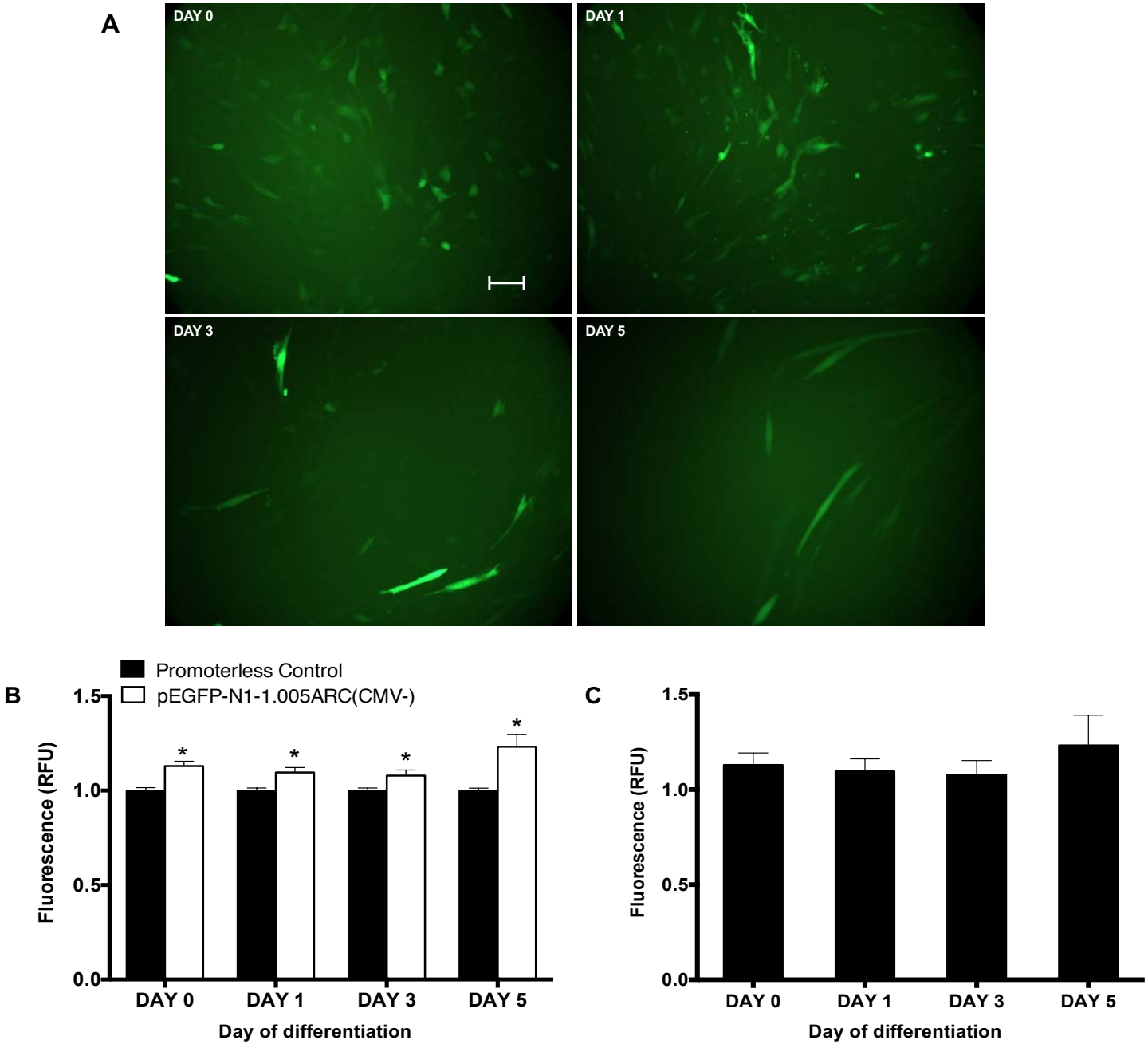


Figure 26: GFP fluorescence in differentiated C2C12 cells transfected with the RAZ-ARC-EFGP plasmid. A) GFP fluorescence was detected under the fluorescent microscope throughout C2C12 cell differentiation. Bar represents 100 μm. B) GFP fluorescence of cells transfected with the promoterless control or the RAZ-ARC-EGFP plasmid across the differentiation period. The GFP fluorescence of cells transfected with the RAZ-ARC-EGFP plasmid was significantly higher than the GFP fluorescence in cells transfected with the promoterless control plasmid at all time points. C) GFP fluorescence of cells transfected with the RAZ-ARC-EGFP across the differentiation period. There was no difference in GFP fluorescence as C2C12 cells differentiated. * = $p<0.05$ compared to promoterless control. (n=6, in triplicate).

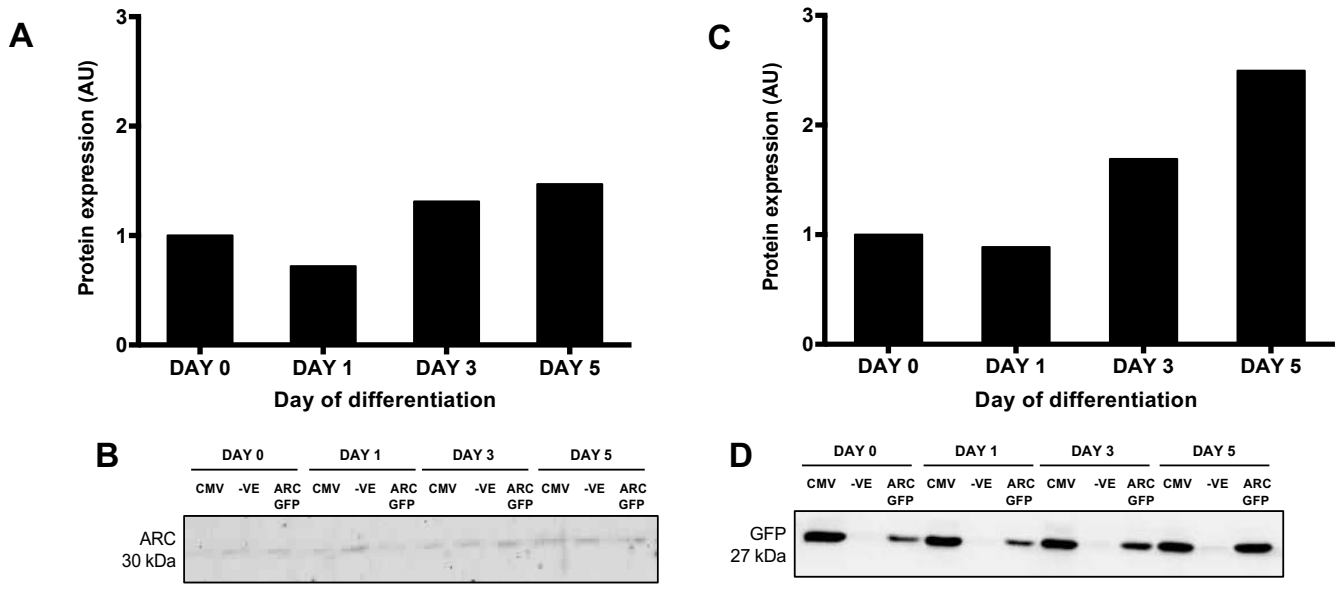


Figure 27: ARC protein levels increased as C2C12 cells differentiated. GFP protein levels increased in differentiating C2C12 cells transfected with the RAZ-ARC-EGFP plasmid. A) Quantification of ARC protein levels. B) Whole cell lysate immunoblot of ARC. C) Quantification of GFP protein levels in cells transfected with the pEGFP-N1-1.005ARC(CMV-) plasmid. D) Whole cell lysate immunoblot of GFP. "CMV" represents cells transfected with the pEGFP-N1 positive control plasmid. "–VE" represented cells transfected with the pEGFP-N1(CMV-) negative control plasmid. "ARC GFP" represents cells transfected with the RAZ-ARC-EGFP plasmid (n=1).

Discussion

GFP reporter systems are commonly used in research as an easy way to determine the expression levels of a protein of interest (96). The purpose of this thesis was to develop a GFP reporter system that would allow for the analysis of ARC promoter activation. ARC is a potent anti-apoptotic protein that is highly upregulated in many cancers and has been associated with the resistance of cancer cells to chemotherapeutic drugs (62,70,72,73). It has been shown that a decrease in ARC protein levels results in an increased sensitivity to apoptosis induced by chemotherapeutic drugs, making ARC an appealing therapeutic target (70,71). A GFP plasmid under the control of the ARC promoter would allow for the examination of ARC expression as cells are incubated in various drugs. Theoretically, an increase or decrease of GFP fluorescence should correlate with an increase or decrease in ARC expression, respectively. GFP presents an advantage over the commonly used luciferase reporter systems for promoter analysis because fluorescence can be measured without lysing the cells and without the addition of other substrates (96,99). This allows for frequent monitoring of GFP fluorescence. The goal of this thesis was to develop a GFP plasmid under the control of the ARC promoter and analyse how GFP fluorescence changed in response to changes in ARC promoter activation. To accomplish this, a region of the ARC promoter was inserted into a GFP reporter plasmid with the expectation that the amount of GFP fluorescence would correlate with the amount of ARC promoter activation. To test this hypothesis, various cell types were transfected with the GFP plasmid containing the ARC promoter region. Following transfection, measures of GFP fluorescence were performed via fluorescent microscopy and plate reader assays, as well as measures of ARC and GFP protein content through Western blots.

Previous studies that have analysed the ARC promoter region have used luciferase based reporter systems (75,80). The luciferase based reporter system is useful for promoter analysis because there is limited background noise, since the light production from a luciferase assay is the result of an enzymatic reaction and does not require an additional light source for excitation (102). This makes the assay highly sensitive because it is able to detect small changes in light production (101). However, the luciferase assay requires the cells to be lysed before analysis (96). The original goal of this project was to monitor the GFP expression response to an increase or decrease in ARC promoter activation in the same set of cells at various time points during incubation in different compounds. Measuring the GFP fluorescence in the same set of cells is important because it decreases the possibility that the changes in GFP fluorescence, and therefore ARC expression, are due to differences in the phenotype of the cells in each plate. Additionally, the fact that the cells do not need to be lysed means that GFP fluorescence can be measured at multiple stages of incubation, which is important in determining the critical time point(s) when GFP fluorescence, and therefore ARC promoter activation, changes over the course of the treatment period. Additionally, the measurement of GFP fluorescence does not require the addition of substrates before analysis (98). This saves time, effort, and money. While the GFP approach provides a number of advantages for the examination of ARC promoter activation, it requires the ARC promoter to be highly activated to produce a sufficient amount of GFP to overcome the autofluorescence of the cell (99). Since fluorescent assays require an additional light source to excite the green fluorescent molecules, cellular components can also be excited and fluoresce in the green light spectrum, resulting in background fluorescence (99). A constitutive promoter is commonly included in GFP reporter plasmids to allow for the sufficient production of GFP to overcome background fluorescence (99). However, it was important for this project that the ARC

promoter be the driver of GFP expression. With that being said, the use of the GFP plasmid driven by the ARC promoter in further analyses, such as a high throughput drug screen, was limited by the small amount of GFP fluorescence detected by the plate reader in C2C12 and MCF7 cells transfected with the RAZ-ARC-EGFP plasmid. Because there was a limited amount of GFP, a significant difference in GFP fluorescence was not detected by the plate reader. In the future, the detection of differences in GFP fluorescence will be vital to determining whether or not a compound is able to increase or decrease ARC promoter activation. This small amount of GFP produced by the RAZ-ARC-EGFP plasmid could be explained by limited activation of the ARC promoter region. Since the GFP reporter plasmid is under the control of the ARC promoter, the amount of GFP being made would correspond to the amount of ARC promoter activation and would theoretically be similar to the amount of ARC protein made (however, it is important to note that not all mRNA is translated into protein). If ARC protein levels are low in comparison to the total protein content of the cell, then GFP expression will also be low and may not be detected by a plate reader. Instead, a more sensitive detection instrument may be needed. Therefore, a plate reader assay may not be sensitive enough to detect changes in GFP fluorescence over a period of time in this experiment. However, this is likely not the case as we used C2C12 and MCF7 cells, which have been shown to have a higher ARC protein content than most other cell lines. Additionally, in this thesis and in previous literature, experiments using the luciferase plasmid under the control of the ARC promoter resulted in luciferase activity, showing that the luciferase plasmids produce the luciferase enzyme in response to ARC promoter activation (Figure 18) (75,80). Therefore, it is clear that the ARC promoter region is being activated at a sufficient level to produce luciferase. Ultimately, further work is required to produce a reporter plasmid that responds to ARC promoter activation.

While the use of GFP for promoter analysis is not common, one study was able to detect GFP fluorescence using a pGL3-EGFP(luc-) plasmid for SV40 promoter analysis in human embryonic lung fibroblasts (HLF) (111). In this previous work, HLF cells were transfected with a pGL3-EGFP(luc-) plasmid driven by the SV40 promoter and a Thy-1.1 (a mouse cell surface antigen) reference plasmid tagged with allophycocyanin (APC) (111). The HLF cells were sorted based on the detection of APC and the cells with a signal intensity above 6 AU were considered successfully transfected (111). This population of cells was then analysed for GFP fluorescence using a flow cytometer (111). Initially sorting cells based on a reference plasmid allowed for greater detection of GFP fluorescence compared to the amount of GFP fluorescence in the entire population. HLF cells have a low transfection efficiency, therefore, the number of cells that contain the GFP expressing plasmid in the entire cell population is low and consequently, so is the amount of GFP fluorescence (111). Sorting the HLF cells based on the reference plasmid limited the HLF cells that were analysed to those that were successfully transfected and therefore a greater amount of GFP fluorescence could be detected (111). However, while a flow cytometer may allow for greater detection of GFP fluorescence, this method is not as quick and simple as the plate reader fluorescence assay. For flow cytometer analysis, cells would need to be isolated and prepared for every test of GFP fluorescence. This would take away the ability to monitor and analyse the same cells over a period of time. Separate cells would be needed for each time point, which would require additional time, materials, and resources. Further, flow cytometry analysis can only be done at certain time points, therefore a critical time point (where there is a change in ARC promoter activity) could be missed. Additionally, analysing the cells using a flow cytometer would limit the number of compounds that could be screened at one time. The plate reader assay would allow for the detection of GFP fluorescence in a 96 (or more) well plate, which would be beneficial when

screening numerous drugs for their effect on ARC promoter activation. Overall, GFP is advantageous for the detection of ARC promoter activation in combination with a high-throughput drug screen, but more research is needed to construct a plasmid that will produce a reporter protein in response to ARC promoter activation that can be detected by plate reader analysis.

Data from several studies suggests that GFP causes cell damage and induces apoptosis in a variety of cell types (112). This can ultimately affect the results of a study, especially those focused on apoptosis. One study revealed that the transduction of GFP led to the generation of ROS and therefore increased the sensitivity of neuroblastoma cells to chemotherapeutic drugs, including DOX (113). However, it is important to note that the lentivirus vector used in this previous work contained a constitutive promoter and therefore the GFP expression was likely high (113). In this thesis, it is unlikely that the amount of GFP that was produced by the FOO-ARC-GFP, FOO-ModARC-GFP, RAZ-ARC-GFP(luc-) and RAZ-ARC-EGFP plasmids was enough to induce toxicity or apoptosis, and account for the poor results observed. However, if a new GFP plasmid is developed that successfully responds to ARC promoter activation and produces a significant amount of GFP, the possibility that GFP can cause cellular damage would need to be taken into consideration. This would be especially true if the plasmid was used for a high-throughput drug screen. The purpose of the pharmaceutical agent that decreases ARC expression is to sensitize cancer cells to apoptosis-inducing chemotherapeutic drugs. If the cancer cells are already sensitive to chemotherapeutic drugs due to GFP, the ability of the agents to decrease ARC promoter activation may be enhanced during the drug screen. Since the ARC protein plays a role in apoptotic signalling, it is possible that ARC may be involved in the increased sensitivity of cells to chemotherapeutic drugs. Luckily, it is possible to test whether or not GFP is having an effect on the sensitivity of cells to the compounds included in a high throughput screen

by transfecting cells with a plasmid that contains the ARC promoter region without the GFP coding sequence. If the results are the same in both the cells transfected with the GFP containing plasmid and the plasmid without the GFP coding sequence, it would be clear that GFP is not a significant problem. Overall, even though GFP allows for a quick and easy detection of ARC promoter activation, its ability to potentially damage the cell would need to be taken into consideration if a GFP plasmid containg the ARC promtoer is used in a high throughput drug screen.

The pGL3-0.75ARC plasmid used in this thesis was constructed by Dr. Roger Foo (University of Singapore) (75). This plasmid was originally used by Wu and colleagues to determine if Ras could activate the 750 base pair ARC promoter region (75). The pGL3-0.75ARC plasmid was transfected into HEK-293 cells (which have low ARC protein content) along with an N-Ras or H-Ras vector (75). To determine if Ras activated the ARC promoter region, luciferase activity was measured using a firefly/*Renilla* luciferase assay (75). The results revealed that the N-Ras and H-Ras vectors were able to activate the ARC promoter region (75). For this thesis, the pGL3-0.75ARC plasmid was modified to encode EGFP instead of luciferase for easier detection. It was subsequently transfected into C2C12 cells which resulted in a limited amount of GFP fluorescence throughout differentiation, even though ARC protein content increases as C2C12 cells differentiate. As this was not the result that we were expecting, the original pGL3-0.75ARC plasmid was tested for its ability to respond to ARC promoter activation by transfecting the original plasmid into MCF7 cells and performing a luciferase assay. MCF7 cells have a high ARC protein content compared to other cancer cell lines and were therefore selected for use in this thesis (Figure 20). Interestingly, the amount of luciferase activity in the MCF7 cells transfected with the original pGL3-0.75ARC plasmid was lower than the luciferase activity seen in cells transfected with the promoterless pGL3 control plasmid (Figure 18). This is different than the results reported

by Wu and colleagues that found an increase in luciferase activity when HEK-293 cells were transfected with the pGL3-0.75ARC plasmid and an N-Ras or H-Ras vector (75). This shows that the 750 base pair ARC promoter region is activated by Ras. To confirm that this was a problem with the pGL3-0.75ARC plasmid and not the luciferase assay protocol, a pGL3-1.005ARC plasmid was obtained from Dr. Olga Razorenova and tested for its ability to respond to ARC promoter activation in MCF7 cells. Previous work by Dr. Olga Razorenova showed that the 1005 base pair ARC promoter region can be activated by hypoxia and the induction of HIF-1α, since luciferase activity increased when TK10 human kidney adenocarcinoma cells were transfected with the pGL3-1.005ARC plasmid and exposed to hypoxic conditions (0.5% oxygen) (80). MCF7 cells transfected with the pGL3-1.005ARC plasmid showed a significant amount of luciferase activity in comparison to the pGL3-0.75ARC plasmid and the promoterless pGL3 control plasmid (Figure 13). This illustrates that the pGL3-0.75ARC plasmid was not responding to ARC promoter activation in MCF7 cells and therefore was not producing luciferase.

Since Wu and colleagues showed that the Ras pathway can stimulate the 750 base pair ARC promoter region and produce luciferase in HEK-293 cells with the addition of N-Ras or H-Ras, it was interesting that the plasmid did not produce the same results in this thesis (75). The MCF7 cells that were used for the luciferase assay in the present thesis have very high ARC protein levels, thus should contain the transcription factors and signaling pathways to promote ARC expression and therefore luciferase activity. Further, this cell line contains high levels of N-Ras, an integral part of the pathway involved in the activation of the ARC promoter region (114). Therefore, we believed that the luciferase assay in this thesis would give similar results to the paper by Wu and colleagues (75). However, it is possible that different ARC transcript variants are present in HEK-293 cells and MCF7 cells. Unfortunately, these same cells have not previously

been transfected with ARC plasmids in the literature. In the paper by Wu and colleagues, the pGL3-0.75ARC plasmid was only transfected into HEK-293 cells to analyse ARC promoter activation and luciferase activity by the Ras signalling pathway (75). In the same paper, MCF7 cells are used for other analyses, however, MCF7 cells were not used for the luciferase assays (75). In the paper by Razorenova and colleagues, the pGL3-1.005ARC plasmid was transfected into 293FT human embryonic kidney cells and TK10 renal cell carcinoma cells (80). ARC promoter activation and luciferase activity was analysed in the 293FT and TK10 cells after cells were exposed to hypoxic conditions (80). It was mentioned in the same paper that the ARC protein content in MCF7 cells increases when hypoxia is induced; however, MCF7 cells were not used for luciferase assays (80). Further, C2C12 cells are not used for ARC promoter analysis in the literature. Therefore, the literature does not provide a clear view on whether different transcript variants may be playing a role in the results of this thesis. Furthermore, the ARC promoter regions incorporated into the plasmids used in this thesis are from two different areas of the ARC gene. Analysis of the sequences showed that the transcriptional start site defined by Dr. Roger Foo (pGL3-0.75ARC) corresponded with the short ARC transcript variants and the transcriptional start site defined by Dr. Olga Razorenova (pGL3-1.005ARC) corresponded with the long ARC transcript variants outlined on the National Institute of Health (NIH) Gene Bank (Figure 9 and 19). However, since it is unknown which transcript variants are present in different tissues, it is possible that the different promoter regions and different transcript variants contributed to the success or failure of the plasmid to produce GFP in different cell types. If this is the case, the plasmid from Dr. Razorenova (pGL3-1.005ARC) may not result in GFP production when transfected into cells containing the short ARC transcript variant given that the ARC promoter region included in this plasmid starts before the transcriptional start site of the short transcript variants. Conversely, the

promoter region present in the plasmid from Dr. Roger Foo (pGL3-0.75ARC) is located after the transcriptional start site of the long transcript variant, and therefore this region may not play a large role in the activation of transcription or contain all the elements necessary for transcription. Ultimately, the different promoter regions and transcript variants could have played a role in the results of this thesis. Therefore, determining the transcript variant present in C2C12 and MCF7 cells would allow us to choose the plasmid with the promoter region that correlates with the ARC variant in the cell. To do so, primers that correspond with the ARC transcript variants can be designed to be used with real-time polymerase chain reaction. This will result in the amplification of the DNA of the transcript variant that is present in the tissue and allow us to establish which promoter region should be included in the reporter plasmid.

Throughout this thesis, cell lines from both mouse and human were used. The C2C12 mouse myoblasts were used because C2C12 cell differentiation was a known way to easily induce ARC protein production over a period of time, thus allowing for the monitoring of GFP expression as ARC protein levels increased. However, the ARC promoters in the plasmids used in this thesis are based on the human ARC sequence. With that being said, mice are commonly used in research because there are many similarities to the human genome (115). The similarities generally lie in the protein-coding regions, while differences are fairly pronounced in non-coding regions (115). However, some important regulatory elements that are contained in the non-coding regions (such as transcription factor binding sites) may still be present in both the mouse and human gene (116). Additionally, there may be some situations where a transcription factor is able to bind to a sequence that is not exactly the same as the established binding site sequence (116). Overall, there may be differences between the non-coding regions of mice and humans, but the important regulatory elements may still remain.

There are some notable differences between the human and mouse ARC gene and promoter. First, the human ARC gene is found on chromosome 16, whereas the ARC gene is found on chromosome 8 in mice (56). Additionally, the human ARC protein is 208 amino acids with a molecular mass of 22.6 kD, whereas the mouse ARC protein is 220 amino acids with a molecular mass of 24.5 kD (56). Interestingly, Figure 11 shows that the ARC protein is larger in C2C12 mouse myoblasts compared to the ARC protein in MCF7 human breast cancer cells. When the entire human ARC sequence was compared to the entire mouse ARC sequence using the NIH Basic Local Alignment Search Tool (BLAST), there were few similarities found in the promoter area. However, the RAZ-ARC-EGFP plasmid, which contains a segment of the human ARC promoter region, responded the best to increasing ARC expression during C2C12 mouse myoblast cell differentiation. The factor(s) responsible for the increase in ARC during C2C12 cell differentiation is not known, therefore, it is possible that this factor(s) may bind to a sequence site that is present within the 1005 base pair human ARC promoter region that is similar to the sequence that the transcription factor(s) binds to in the mouse ARC promoter region. This could be explained by the fact that some regulatory elements in the promoter regions of human and mouse genes are conserved, or that the transcription factor binding sequences do not need to completely align with the established transcription factor binding sites (116). Therefore, the human ARC promoter in the RAZ-ARC-EGFP plasmid may have been activated by the factor(s) responsible for C2C12 mouse myoblast differentiation to produce GFP.

Overall, a GFP reporter plasmid is a valuable tool to use in combination with a high throughput drug screen because it allows the gene or promoter of interest to be analysed on numerous occasions over a period of time on the same set of cells. ARC is a potent anti-apoptotic protein that is highly upregulated in cancer cells and is an attractive therapeutic target because it

has been shown to contribute to the resistance of cancer cells to chemotherapeutic drugs (62,70,72,73). However, the results of this thesis show that more work is required to develop an effective reporter plasmid driven by the ARC promoter. Since the fluorescent reporter system presents many advantages over the luciferase reporter system, it may be beneficial to insert the ARC promoter region into a red fluorescent protein (RFP) plasmid. Autofluorescence of the cell typically occurs in the green light spectrum, therefore, some background noise may be removed by moving to an RFP plasmid (117). Additionally, given that there are different ARC transcript variants, it would be valuable to determine which variant is present in the cell types that will be used so the correct promoter region can be inserted into the plasmid. Ultimately, there is more research needed to develop an effective reporter plasmid to monitor ARC expression levels.

Limitations

There were a variety of limitations that impacted the results of this thesis. First, the plasmids used in this thesis are manufactured pieces of circular DNA. While using reporter plasmids with portions of genes is a widely used method for gene expression and promoter analysis, it may not allow researchers to gain insight into what is actually occurring in the cell because the portion of the gene included in the plasmid is not in the context of the whole genome (118). Therefore, some vital components (such as transcription factor binding sites or enhancer sequences) may not be present (118). In terms of promoter analysis and reporter plasmids, the portion of the promoter region taken out of the context of the genome may not have the same effect on the promotion of transcription (118). It is possible that the regulation of the promoter region may also be altered when it is inserted into a plasmid (118). Therefore, the promoter region in the reporter plasmid may not respond to the activating elements in the same

way it would in the genome and ultimately would not produce the expected reporter protein (118). Additionally, it is possible that all the elements necessary for promoter activation are not present within the portion of the promoter region in the plasmids (118). It has been shown that there are other areas of the genome that are important for the activation of transcription other than the promoter region. Enhancer sequences, for example, bind proteins that are important to the interaction of the elements involved in transcription (119). Ultimately, the fact that the ARC promoter region was taken out of the context of the genome and inserted into a GFP reporter plasmid may have limited the amount of GFP that was produced due to a change in promoter regulation, and therefore limited the results of this thesis.

Transfection of the ARC plasmids into a variety of cell lines was a critical component of this thesis. In this thesis, cells were transfected using both cationic lipid and cationic polymer transfection methods. However, this method limited the cell lines that could be used. Because the primary human myoblasts died following transfection, C2C12 mouse myoblasts were used in this thesis. Additionally, some cell lines have a low transfection efficiency. Therefore, the packaged plasmid DNA could not easily enter the cell and be expressed. Even though the manufacturer's transfection protocols for both Lipofectamine2000 and jetPrime were manipulated to include varying amounts of plasmid DNA and reagents, the transfection method was a problem throughout this thesis.

Future directions

While this project may ultimately result in the discovery of a drug that can be used to decrease ARC protein levels in cancer cells, there are some aspects that should be considered before moving forward. First, a fluorescent reporter system was used for this project to allow for

the simple detection of a change in promoter activation. With this system, we hypothesized we would be able to incubate the cells in various drugs and simply run a plate reader assay to determine whether or not the fluorescence decreased. However, it is possible that a GFP reporter system may not be powerful enough for promoter analysis, especially with the ARC promoter. Further, the autofluorescence of the cell that occurs in the green light spectrum may be limiting the detected GFP fluorescence. In order to test whether there is a problem with the activation of the ARC promoter or the detection of GFP, the luciferase plasmids containing the ARC promoter and the GFP plasmids containing the ARC promoter can be tested in tandem. If there is a problem with the activation of the ARC promoter, both the luciferase assay and the GFP fluorescence assay should be affected. However, if there is a problem with the detection of GFP, only the GFP fluorescence assay should be affected. Additionally, it would be interesting to examine the amount of fluorescence detected by a plate reader when a RFP plasmid is driven by the ARC promoter. Additionally, it may be beneficial to explore other simple options, such as secreted alkaline phosphatase (SEAP). Similar to other reporter genes, a segment of DNA is inserted into a SEAP reporter plasmid. The activation of this segment of DNA results in the production of SEAP and this is secreted into the cell culture medium (98). SEAP can be detected using a chemiluminescent assay (96). This method is easy to use and would be appropriate for the analysis of promoter activation over a period of time as it does not require the cells to be lysed (96). The cell media is collected and analysed for the presence of SEAP (96).

Second, it is possible that different transcript variants are present in different cell types. It would be beneficial to know which transcript variant is present in the cell in order to choose the appropriate promoter region to use in the reporter plasmid. While it is not possible to insert the entire genome into a reporter plasmid, using the correct transcript variant for the cell line may

result in a more successful experiment. Real-time polymerase chain reaction will allow us to determine which transcript variant is present in the cell lines examined.

Another method to increase ARC levels would be to co-transfect cells with the transcription factors that are known to drive ARC transcription along with the reporter plasmid containing the ARC promoter region. This would provide us with an additional model to analyse ARC promoter activation and the response of the reporter plasmid.

To further understand ARC transcription, ARC mRNA expression can be measured. This will provide us with information regarding the amount of ARC mRNA that is being made and help us better understand the levels of the reporter protein and its relationship with ARC transcription.

Finally, if a successful reporter plasmid is made, there are numerous applications. This plasmid can be used in various cell types to track the levels of ARC expression. Since ARC appears to play an important role in skeletal and cardiac muscle, the plasmid could be used to enhance our understanding of how different conditions, such as aging or disease, affect ARC levels. Additionally, this plasmid may be able to help researchers understand the transcriptional regulation of ARC and the transcription factors involved. With a successful reporter plasmid, we may be able to use this plasmid to find a drug that would decrease ARC expression and hopefully increase the sensitivity of cancer cells to apoptosis-inducing chemotherapeutic drugs. Transfected cells can be incubated with various drugs for a period of time and the ARC expression levels can be tracked using a plate reader assay. Additionally, there is a chance that a drug may increase ARC expression. This could have implications in diseases where too much apoptosis is occurring, such as Alzheimer's and heart disease.

References

1. Saikumar P, Dong Z, Mikhailov V, Denton M, Weinberg JM, Venkatachalam MA. Apoptosis: Definition, mechanisms, and relevance to disease. Am J Med. 1999;107:489–506.
2. Fuchs Y, Steller H. Programmed cell death in animal development and disease. Cell. 2011;147:742–58.
3. Ziegler U. Morphological Features of Cell Death. News Physiol Sci. 2004;19:124–8.
4. Nuñez G, Benedict M a, Hu Y, Inohara N. Caspases: the proteases of the apoptotic pathway. Oncogene. 1998;17:3237–45.
5. Danial NN, Korsmeyer SJ. Cell Death: Critical Control Points. Cell. 2004;116:205–19.
6. Kroemer G, Reed JC. Mitochondrial control of cell death. Nat Med. 2000;6:513–9.
7. Chipuk JE, Bouchier-Hayes L, Green DR. Mitochondrial outer membrane permeabilization during apoptosis: the innocent bystander scenario. Cell Death Differ. 2006;13:1396–402.
8. Chipuk JE, Moldoveanu T, Llambi F, Parsons MJ, Green DR. The BCL-2 Family Reunion. Mol Cell. 2010;37:299–310.
9. Luna-Vargas MPA, Chipuk JE. The deadly landscape of pro-apoptotic BCL-2 proteins in the outer mitochondrial membrane. FEBS J. 2016;1–14.
10. Wei MC, Zong W-X, Cheng EH-Y, Lindsten T, Panoutsakopoulou V, Ross AJ, et al. Proapoptotic BAX and BAK: A Requisite Gateway to Mitochondrial Dysfunction and Death. Science (80-). 2001;292:727–30.
11. Chipuk JE, Green DR. How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? Trends Cell Biol. 2008. page 157–64.
12. Shamas-Din A, Brahmbhatt H, Leber B, Andrews DW. BH3-only proteins: Orchestrators of apoptosis. Biochim Biophys Acta - Mol Cell Res. 2011;1813:508–20.
13. Giam M, Huang DCS, Bouillet P. BH3-only proteins and their roles in programmed cell death. Oncogene. 2008;27 Suppl 1:S128–36.
14. Happo L, Strasser A, Cory S. BH3-only proteins in apoptosis at a glance. J Cell Sci. 2012;125:1081–7.
15. Adams JM, Cory S. The Bcl-2 apoptotic switch in cancer development and therapy. Oncogene. 2007;26:1324–37.
16. Lomonosova E, Chinnadurai G. BH3-only proteins in apoptosis and beyond: an overview. Oncogene. 2008;27 Suppl 1:S2-19.
17. Westphal D, Kluck RM, Dewson G. Building blocks of the apoptotic pore: how Bax and Bak are activated and oligomerize during apoptosis. Cell Death Differ. 2014;21:196–205.
18. Tait SWG, Green DR. Mitochondria and cell death: outer membrane permeabilization and beyond. Nat Rev Mol Cell Biol. 2010;11:621–32.
19. Wang JX, Li Q, Li PF. Apoptosis repressor with caspase recruitment domain contributes to chemotherapy resistance by abolishing mitochondrial fission mediated by dynamin-related protein-1. Cancer Res. 2009;69:492–500.
20. van Loo G, Saelens X, van Gurp M, MacFarlane M, Martin SJ, Vandenabeele P. The role of mitochondrial factors in apoptosis: a Russian roulette with more than one bullet. Cell Death Differ. 2002;9:1031–42.
21. Galluzzi L, Kepp O, Trojel-Hansen C, Kroemer G. Non-apoptotic functions of apoptosis-regulatory proteins. EMBO Rep. 2012;13:322–30.

22. Saleh A, Srinivasula SM, Acharya S, Fishel R, Alnemri ES. Cytochrome c and dATP-mediated oligomerization of Apaf-1 is a prerequisite for procaspase-9 activation. J Biol Chem. 1999;274:17941–5.
23. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, et al. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell. 1997;91:479–89.
24. Chen Q, Gong B, Almasan A. Distinct stages of cytochrome c release from mitochondria: evidence for a feedback amplification loop linking caspase activation to mitochondrial dysfunction in genotoxic stress induced apoptosis. Cell Death Differ. 2000;7:227–33.
25. Chen Q, Chai Y-C, Mazumder S, Jiang C, Macklis RM, Chisolm GM, et al. The late increase in intracellular free radical oxygen species during apoptosis is associated with cytochrome c release, caspase activation, and mitochondrial dysfunction. Cell Death Differ. 2003;10:323–34.
26. Candé C, Vahsen N, Kouranti I, Schmitt E, Daugas E, Spahr C, et al. AIF and cyclophilin A cooperate in apoptosis-associated chromatinolysis. Oncogene. 2004;23:1514–21.
27. Li LY, Luo X, Wang X. Endonuclease G is an apoptotic DNase when released from mitochondria. Nature. 2001;412:95–9.
28. Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell. 2000;102:33–42.
29. Guicciardi ME, Gores GJ. Life and death by death receptors. FASEB J. 2009;23:1625–37.
30. Nam YJ, Mani K, Ashton AW, Peng CF, Krishnamurthy B, Hayakawa Y, et al. Inhibition of both the extrinsic and intrinsic death pathways through nonhomotypic death-fold interactions. Mol Cell. 2004;15:901–12.
31. Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell. 1998;94:491–501.
32. Stennicke HR, Jurgensmeier JM, Shin H, Deveraux Q, Wolf BB, Yang X, et al. Pro-caspase-3 Is a Major Physiologic Target of Caspase-8. J Biol Chem. 1998;273:27084–90.
33. Rutkowski DT, Kaufman RJ. A trip to the ER: Coping with stress. Trends Cell Biol. 2004;14:20–8.
34. Rizzuto R, Pinton P, Ferrari D, Chami M, Szabadkai G, Magalhães PJ, et al. Calcium and apoptosis: facts and hypotheses. Oncogene. 2003;22:8619–27.
35. Nakagawa T, Yuan J. Cross-talk between Two Cysteine Protease Families: Activation of Caspase-12 by Calpain in Apoptosis. J Cell Biol. 2000;150:887–94.
36. Yoneda T, Imaizumi K, Oono K, Yui D, Gomi F, Katayama T, et al. Activation of Caspase-12, an Endoplastic Reticulum (ER) Resident Caspase, through Tumor Necrosis Factor Receptor-associated Factor 2-dependent Mechanism in Response to the ER Stress*. J Biol Chem. 2001;276:13935–40.
37. Martinez JA, Zhang Z, Stanislav SI, Hayes RL, Wang KK, Larner SF. Calpain and caspase processing of caspase-12 contribute to the ER stress-induced cell death pathway in differentiated PC12 cells. Apoptosis. 2010;15:1480–93.
38. Lee W, Kim DH, Boo JH, Kim YH, Park I-S, Mook-Jung I. ER stress-induced caspase-12 activation is inhibited by PKC in neuronal cells. Apoptosis. 2005;10:407–15.
39. Morishima N, Nakanishi K, Takenouchi H, Shibata T, Yasuhiko Y. An Endoplasmic Reticulum Stress-specific Caspase Cascade in Apoptosis Cytochrome c-Independent Activation of Caspase-9 by Caspase-12*. J Biol Chem. 2002;277:34287–94.

40. Sano R, Reed JC. ER stress-induced cell death mechanisms. Biochim Biophys Acta - Mol Cell Res. 2013;1833:3460–70.
41. Oyadomari S, Mori M. Roles of CHOP/GADD153 in endoplasmic reticulum stress. Cell Death Differ. 2004;11:381–9.
42. Contreras L, Drago I, Zampese E, Pozzan T. Mitochondria: The calcium connection. Biochim Biophys Acta - Bioenerg. 2010;1797:607–18.
43. Santo-Domingo J, Demaurex N. Calcium uptake mechanisms of mitochondria. Biochim Biophys Acta - Bioenerg. 2010;1797:907–12.
44. Crompton M. The mitochondrial permeability transition pore and its role in cell death. Biochem J. 1999;341:233–49.
45. Orrenius S, Zhivotovsky B, Nicotera P. Calcium: Regulation of cell death: the calcium–apoptosis link. Nat Rev Mol Cell Biol. 2003;4:552–65.
46. Meek DW. The p53 response to DNA damage. DNA Repair (Amst). 2004;3:1049–56.
47. Schuler M, Green DR. Mechanisms of p53-dependent apoptosis. Biochem Soc Trans. 2001;29.
48. Nakano K, Vousden KH. PUMA, a Novel Proapoptotic Gene, Is Induced by p53. Mol Cell. 2001;7:683–94.
49. Lin Y, Ma W, Benchimol S. Pidd, a new death-domain-containing protein, is induced by p53 and promotes apoptosis. Nat Genet. 2000;26:122–7.
50. Bouchier-Hayes L, Green DR. Caspase-2: the orphan caspase. Cell Death Differ. 2012;19:51–7.
51. Baptiste-Okoh N, Barsotti AM, Prives C. A role for caspase 2 and PIDD in the process of p53-mediated apoptosis. Proc Natl Acad Sci U S A. 2008;105:1937–42.
52. Tinel A, Tschopp J. The PIDDosome, a Protein Complex Implicated in Activation of Caspase-2 in Response to Genotoxic Stress. Science (80-). 2004;304.
53. Guo Y, Srinivasula SM, Druilhe A, Fernandes-Alnemri T, Alnemri ES. Caspase-2 Induces Apoptosis by Releasing Proapoptotic Proteins from Mitochondria*. J Biol Chem. 2002;277:13430–7.
54. Green DR, Kroemer G. Cytoplasmic functions of the tumour suppressor p53. Nature. 2009;458:1127–30.
55. Koseki T, Inohara N, Chen S, Nez G. ARC, an inhibitor of apoptosis expressed in skeletal muscle and heart that interacts selectively with caspases. Genet Commun by James V Neel. 1998;95:5156–60.
56. Ludwig-Galezowska AH, Flanagan L, Rehm M. Apoptosis repressor with caspase recruitment domain, a multifunctional modulator of cell death. J Cell Mol Med. 2011;15:1044–53.
57. Jang T, Kim SH, Jeong J-H, Kim S, Kim YG, Park HH, et al. Crystal structure of caspase recruiting domain (CARD) of apoptosis repressor with CARD (ARC) and its implication in inhibition of apoptosis. Sci Rep. 2015;5:9847.
58. Li Y-Z, Lu D-Y, Tan W-Q, Wang J-X, Li P-F. p53 initiates apoptosis by transcriptionally targeting the antiapoptotic protein ARC. Mol Cell Biol. 2008;28:564–74.
59. Jo D-G, Jun J-I, Chang J-W, Hong Y-M, Song S, Cho D-H, et al. Calcium binding of ARC mediates regulation of caspase 8 and cell death. Mol Cell Biol. 2004;24:9763–70.
60. McKimpson WM, Weinberger J, Czerski L, Zheng M, Crow MT, Pessin JE, et al. The apoptosis inhibitor ARC alleviates the ER stress response to promote β-cell survival. Diabetes. 2013;62:183–93.

61. Foo RS-Y, Nam Y-J, Ostreicher MJ, Metzl MD, Whelan RS, Peng C-F, et al. Regulation of p53 tetramerization and nuclear export by ARC. Proc Natl Acad Sci U S A. 2007;104:20826–31.
62. Wang M, Qanungo S, Crow MT, Watanabe M, Nieminen AL. Apoptosis repressor with caspase recruitment domain (ARC) is expressed in cancer cells and localizes to nuclei. FEBS Lett. 2005;579:2411–5.
63. Loan Le TY, Mardini M, Howell VM, Funder JW, Ashton AW, Mihailidou AS. Low-Dose Spironolactone Prevents Apoptosis Repressor With Caspase Recruitment Domain Degradation During Myocardial Infarction. Hypertension. 2012;59:1164–9.
64. Abmayr S, Crawford RW, Chamberlain JS. Characterization of ARC, apoptosis repressor interacting with CARD, in normal and dystrophin-deficient skeletal muscle. Hum Mol Genet. 2003;13:213–21.
65. Hong Y-M, Jo D-G, Lee J-Y, Chang J-W, Nam J-H, Noh JY, et al. Down-regulation of ARC contributes to vulnerability of hippocampal neurons to ischemia/hypoxia. FEBS Lett. 2003;543:170–3.
66. Ekhterae D, Lin Z, Lundberg MS, Crow MT, Brosius FC, Núñez G. ARC Inhibits Cytochrome c Release From Mitochondria and Protects Against Hypoxia-Induced Apoptosis in Heart-Derived H9c2 Cells. Circ Res. 1999;85:e70-7.
67. Donath S, Li P, Willenbockel C, Al-Saadi N, Gross V, Willnow T, et al. Apoptosis Repressor With Caspase Recruitment Domain Is Required for Cardioprotection in Response to Biomechanical and Ischemic Stress. Circulation. 2006;113:1203–12.
68. Gustafsson ÅB, Sayen MR, Williams SD, Crow MT, Gottlieb RA. TAT Protein Transduction Into Isolated Perfused Hearts. Circulation. 2002;106.
69. Xiao R, Ferry AL, Dupont-Versteegden EE. Cell death-resistance of differentiated myotubes is associated with enhanced anti-apoptotic mechanisms compared to myoblasts. Apoptosis. 2011;16:221–34.
70. Wang J, Feng C, He Y, Ding W, Sheng J, Arshad M, et al. Phosphorylation of apoptosis repressor with caspase recruitment domain by protein kinase CK2 contributes to chemotherapy resistance by inhibiting doxorubicin induced apoptosis. Oncotarget. 2015;6:27700–13.
71. Carter BZ, Qiu YH, Zhang N, Coombes KR, Mak DH, Thomas DA, et al. Expression of ARC (apoptosis repressor with caspase recruitment domain), an antiapoptotic protein, is strongly prognostic in AML. Blood. 2011;117:780–7.
72. Mercier I, Vuolo M, Madan R, Xue X, Levalley AJ, Ashton AW, et al. ARC, an apoptosis suppressor limited to terminally differentiated cells, is induced in human breast cancer and confers chemo- and radiation-resistance. Cell Death Differ. 2005;12:682–6.
73. Mercier I, Vuolo M, Jasmin JF, Medina CM, Williams M, Mariadason JM, et al. ARC (apoptosis repressor with caspase recruitment domain) is a novel marker of human colon cancer. Cell Cycle. 2008;7:1640–7.
74. Mak PY, Mak DH, Mu H, Shi Y, Ruvolo P, Ruvolo V, et al. Apoptosis repressor with caspase recruitment domain is regulated by MAPK/PI3K and confers drug resistance and survival advantage to AML. Apoptosis. 2014;19:698–707.
75. Wu L, Nam YJ, Kung G, Crow MT, Kitsis RN. Induction of the apoptosis inhibitor ARC by Ras in human cancers. J Biol Chem. 2010;285:19235–45.
76. Oikawa T. ETS transcription factors: possible targets for cancer therapy. Cancer Sci. 2004;95:626–33.

77. Wasylyk B, Hagman J, Gutierrez-Hartmann A. Ets transcription factors: nuclear effectors of the Ras-MAP-kinase signaling pathway. Trends Biochem Sci. 1998;23:213–6.
78. Carter BZ, Andreeff M. Targeting AML stem/progenitor cells by combinational therapy with SMAC mimetics and demethylating agents. Stem Cell Epigenetics. 2015;2.
79. Sun S-C. Non-canonical NF-κB signaling pathway. Cell Res. 2011;21:71–85.
80. Razorenova O V, Castellini L, Colavitti R, Edgington LE, Nicolau M, Huang X, et al. The apoptosis repressor with a CARD domain (ARC) gene is a direct hypoxia-inducible factor 1 target gene and promotes survival and proliferation of VHL-deficient renal cancer cells. Mol Cell Biol. 2014;34:739–51.
81. Rohn TT. The role of caspases in Alzheimer's disease; potential novel therapeutic opportunities. Apoptosis. 2010;15:1403–9.
82. Lee Y, Gustafsson ÅB. Role of apoptosis in cardiovascular disease. Apoptosis. 2009;14:536–48.
83. Wong RSY. Apoptosis in cancer: from pathogenesis to treatment. J Exp Clin Cancer Res. 2011;30:1–14.
84. Hanahan D, Weinberg RA. The Hallmarks of Cancer. Cell. 2000;100:57–70.
85. Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. Cell. 2011;144:646–74.
86. Weinstein IB, Joe AK. Mechanisms of Disease: oncogene addiction—a rationale for molecular targeting in cancer therapy. Nat Clin Pract Oncol. 2006;3:448–57.
87. Wang Q, Li A, Wang H, Wang J. Knockdown of apoptosis repressor with caspase recruitment domain (ARC) increases the sensitivity of human glioma cell line U251MG to VM-26. Int J Clin Exp Pathol. 2012;5:555–61.
88. Muller PAJ, Vousden KH. p53 mutations in cancer. Nat Cell Biol. 2013;15:2–8.
89. Moll UM, Riou G, Levine AJ. Two distinct mechanisms alter p53 in breast cancer: mutation and nuclear exclusion. Proc Natl Acad Sci U S A. 1992;89:7262–6.
90. Nikolaev AY, Li M, Puskas N, Qin J, Gu W. Parc: A Cytoplasmic Anchor for p53. Cell. 2003;112:29–40.
91. Delbridge ARD, Grabow S, Strasser A, Vaux DL. Thirty years of BCL-2: translating cell death discoveries into novel cancer therapies. Nat Rev Cancer. 2016;16:99–109.
92. Fesik SW. Promoting apoptosis as a strategy for cancer drug discovery. Nat Rev Cancer. 2005;5:876–85.
93. Tacar O, Sriamornsak P, Dass CR. Doxorubicin: an update on anticancer molecular action, toxicity and novel drug delivery systems. J Pharm Pharmacol. 2013;65:157–70.
94. Force T, Kolaja KL. Cardiotoxicity of kinase inhibitors: the prediction and translation of preclinical models to clinical outcomes. Nat Rev Drug Discov. 2011;10:111–26.
95. Mullard A. Pioneering apoptosis-targeted cancer drug poised for FDA approval. Nat Rev Drug Discov. 2016;15:147–9.
96. Naylor LH. Reporter gene technology: the future looks bright. Biochem Pharmacol. 1999;58:749–57.
97. Misteli T, Spector DL. Applications of the green fluorescent protein in cell biology and biotechnology. Nat Biotechnol. 1997;15:961–4.
98. Schenborn E, Groskreutz D. Reporter gene vectors and assays. Mol Biotechnol. 1999;13:29–44.
99. Tsien RY. THE GREEN FLUORESCENT PROTEIN. Annu Rev Biochem. 1998;67:509–44.

100. Vidugiriene J, Schagat T, Garvin D, Vidugris G. Getting the Most from Your Transfections: Increasing Throughput and Sensitivity. Promega Notes. 2008;1:3–5.
101. Ghim CM, Lee SK, Takayama S, Mitchell RJ. The art of reporter proteins in science: Past, present and future applications. BMB Rep. 2010;43:451–60.
102. Allard STM. Bioluminescent reporter genes. Promega Corp Web site. 2008;54:350–3.
103. Canadian Cancer Society's Advisory Committee on Cancer Statistics. Canadian Cancer Statistics 2016. Toronto, ON; 2016.
104. Siebring-van Olst E, Vermeulen C, de Menezes RX, Howell M, Smit EF, van Beusechem VW. Affordable Luciferase Reporter Assay for Cell-Based High-Throughput Screening. J Biomol Screen. 2012;18:453–61.
105. Xie G, Zhan J, Tian Y, Liu Y, Chen Z, Ren C, et al. Mammosphere cells from high-passage MCF7 cell line show variable loss of tumorigenicity and radioresistance. Cancer Lett. 2012;316:53–61.
106. Sandri M, Sandri C, Gilbert A, Skurk C, Calabria E, Picard A, et al. Foxo Transcription Factors Induce the Atrophy-Related Ubiquitin Ligase Atrogin-1 and Cause Skeletal Muscle Atrophy. Cell. 2004;117:399–412.
107. Lu D, Liu J, Jiao J, Long B, Li Q, Tan W, et al. Transcription factor Foxo3a prevents apoptosis by regulating calcium through the apoptosis repressor with caspase recruitment domain. J Biol Chem. 2013;288:8491–504.
108. Andronikou N, Yu X, Wei J, Sridharan M, Quintanilla R, Lakshmipathy U, et al. Lipofectamine ® 3000—efficient, reproducible transfection for biologically-relevant cell models. Life Technologies Corporation; 2014.
109. Ao J e., Kuang L hong, Zhou Y, Zhao R, Yang C ming. Hypoxia-inducible Factor 1 regulated ARC expression mediated hypoxia induced inactivation of the intrinsic death pathway in p53 deficient human colon cancer cells. Biochem Biophys Res Commun. 2012;420:913–7.
110. Wu D, Yotnda P. Induction and testing of hypoxia in cell culture. J Vis Exp. 2011;e2899.
111. Ducrest A-L, Amacker M, Lingner J, Nabholz M. Detection of promoter activity by flow cytometric analysis of GFP reporter expression. Nucleic Acids Res. 2002;30:e65.
112. Ansari AM, Ahmed AK, Matsangos AE, Lay F, Born LJ, Marti G, et al. Cellular GFP Toxicity and Immunogenicity: Potential Confounders in in Vivo Cell Tracking Experiments. Stem Cell Rev Reports. 2016;12:553–9.
113. Goto H, Yang B, Petersen D, Pepper KA, Alfaro PA, Kohn DB, et al. Transduction of green fluorescent protein increased oxidative stress and enhanced sensitivity to cytotoxic drugs in neuroblastoma cell lines. Mol Cancer Ther. 2003;2.
114. Graham KA, Richardson CL, Minden MD, Mcf- CCL, Trent JM, Buick RN. Varying Degrees of Amplification of the N- ras Oncogene in the Human Breast Cancer Cell Line MCF-7 Varying Degrees of Amplification of the N-ras Oncogene in the Human Breast. Cancer Res. 1985;45:2201–5.
115. Chinwalla AT, Cook LL, Delehaunty KD, Fewell GA, Fulton LA, Fulton RS, et al. Initial sequencing and comparative analysis of the mouse genome. Nature. 2002;420:520–62.
116. Dermitzakis ET, Clark AG. Evolution of Transcription Factor Binding Sites in Mammalian Gene Regulatory Regions: Conservation and Turnover. Mol Biol Evol. 2002;19:1114–21.
117. Billinton N, Knight AW. Seeing the Wood through the Trees: A Review of Techniques for Distinguishing Green Fluorescent Protein from Endogenous Autofluorescence. Anal

Biochem. 2001;291:175–97.
118. Dale JW, Von Schantz M. From Genes to Genomes: Concepts and Applications of DNA Technology. John Wiley & Sons, Ltd.; 2002.
119. Maston GA, Landt SG, Snyder M, Green MR. Characterization of Enhancer Function from Genome-Wide Analyses. Annu Rev Genomics Hum Genet. 2012;13:29–57.

Appendix

ARC promoter region

First 7 amino acids of ARC protein

HindIII restriction site

GFP

gtcrrtmggtgcagamtttctctatcgataggtaccgagctcttacgcgtgctagcccgg
gctcgagcggccgccagtgtgatggatatctgcagaattcgccctttgtaaaacgacggc
cagttatcagggtcaaggtaaggggagaggggcagggaggctctcagcactttgtgctct
gggttcagaggggactggaggatagagtatggaagcagggagctctgatcctgggttctg
cagggccaaagcaggaggcagaaggctggacttagtaagtggtgaggatggtgagagaaa
tattcagcagtcagactggaaaggacttgtctgcaagctgggtgtggagtatcagcatca
atcctaggtttttctgcctggttaggtggggaagcagatttgagagaaagaccttagttt
ggataaacggggtctgaactcgcctcggatttgccgggggtggagctcaggacgtcctgg
ttggggagggcattcagagagtagatgccagtcctgggaaaggcaggggaggagaggaga
gccacggctgacgcttggggacagaaggaggagcctgaggaggagacaggacagagcgtc
tggagaggcaggaggacaccgagttccccgtgttggcctccaggtcctgtgcttgcggag
ccgtccggcggctgggatcgaggtgaacttaaaccccagcctgggtgttcactgggggca
ttgagggagtggtcagaggcggcgagggcagcggggagggcaacccccccattacttctct
ccccctttccccatgcagccccgacaatgggcaacgcgcacgagcggaagcttgccaccat
ggtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcgagctggacgg
cgacgtaaacggccacaagttcagcgtgtccgsgagggcgagggcgatgccacctacggc
aagctgaccctgaagttcatctgcaccaccggcaagctgcccgtgcctggaccacctcgt
gaccaccctgacctacgsgtgcagtgctcagcgctacccgacacatgaagcagcacgact
ctcagtcgcatgcccgagctacgtcaggagcgcacatctctcagacgacgcactacagac
gsacgaggtgagtcgaggcgaccctgtgacgcatcgactkaggcatcgactcagagacgc
actctggcaagctgatactwcaagtcwmaagtc

Appendix Figure 1: Sequencing results of pGL3-0.75MODARC-GFP(luc-) plasmid. Sequenced using the RV3P primer.

pGL3-basic

KpnI restriction site

HindIII restriction site

GFP

gtcattmgtgcmkaamtttctctatcgataggtaccaagcttgccaccatggtgagcaag
ggcgaggagctgttcaccggggtggtgcccatcctggtcgagctggacggcgacgtaaac
ggccacaagttcagcgtgtccggcgagggcgagggcgatgccacctacggcaagctgacc
ctgaagttcatctgcaccaccggcaagctgcccgtgccctggcccaccctcgtgaccacc
ctgacctacggcgtgcagtgcttcagccgctaccccgaccacatgaagcagcacgacttc
ttcaagtccgccatgcccgaaggctacgtccaggagcgcaccatcttcttcaaggacgac
ggcaactacaagacccgcgccgaggtgaagttcgagggcgacaccctggtgaaccgcatc
gagctgaagggcatcgacttcaaggaggacggcaacatcctggggcacaagctggagtac
aactacaacagccacaacgtctatatcatggccgacaagcagaagaacggcatcaaggtg
aacttcaagatccgccacaacatcgaggacggcagcgtgcagctcgccgaccactaccag
cagaacaccccatcggcgacggccccgtgctgctgcccgacaaccactacctgagcacc
cagtccgccctgagcaaagaccccaacgagaagcgcgatcacatggtcctgctggagttc
gtgaccgccgccgggatcactctcggcatggacgagctgtacaagtaaagcggccgcgac
gctagagtcggggcggccggccgcttcgagcagacatgataagatacattgatgagtttg
gacaaaccacaactagaatgcagtgaaaaaaatgctttatttgtgaaatttgtgatgcta
ttgctttatttgtaaccattataagctgcaataaacaagttaacaacaacaattgcattc
attttatgtttcagtcaggarwgtttttgaaagtttttttaaagcargtaaaacctctaca
atgtggtaaatcgataaggatccgtcgaccgatgcccttgagagcttcaaccagtcagct
ccttccggtggcgcggggcakactatcgtccgccgcmmttatgactkgtcttctttatca
tgcaacmsgaggamaggtrccgacagcgctattrcgmgttctgcattcatgactgcttgc
actcgggyagtcgactgcgcgagcggatwcacgtctcwgttaagactgtgt

Appendix Figure 2: Sequencing results of the pGL3-GFP(luc-) promoterless control plasmid. Sequenced using the RV3P primer.

Transfection of MCF-7 with Lipofectamine 2000
(Maartje Vogel version 120903)
Please check http://www.nki.nl/nkidep/vansteensel for updated versions of this protocol.

One day before transfection

- Harvest cells from exponential phase.
- Seed 4×10^5 cells per well (of a 6-well plate) in 2 ml DMEM complete medium (10% FCS + antibiotics (e.g pen/strep)).
- Rock the plate after seeding.

Cells should be ~90% confluent at the time of transfection.

Day of transfection
Consider the following (control) transfections:

… x Plasmid(s) encoding the Dam protein
… x Plasmid(s) encoding the Dam-fusion protein (your protein of interest fused to Dam).

Controls:
1 x pH2B-GFP (to determine the transfection efficienty by FACS).
2 x no plasmid (control in methylation PCR and neg. control for FACS).

1 x pIND-(V5)-EcoDam and
1 x pIND-Cbx1-V5-EcoDam (serve as a positive control in the methyl-PCR and hybridizations).

1-2 x pIND (empty vector, serves as a control in the methyl PCR).

- For each transfection sample, prepare in separate 1.5 ml tubes:
  1) 2 μg DNA in 100 μl Opti-MEM-1 (RT).
  2) 6 μl Lipofectamine 2000 in 100 μl Opti-MEM-1 (RT). Mix the Lipofectamine gently before use.
- Incubate 5 min. at RT (proceed to next step within 30 min. after diluting the lipofectamine)
- Combine the two solutions, mix gently and incubate for 20 min. at RT (The mixture may appear cloudy).
- In the mean time: wash the cells with DMEM (w/o serum and antibiotics).
- After the 20 min. incubation: add 800 μl DMEM (w/o serum and antibiotics) to the DNA-Lipofectamine mixture, mix gently and add this to the cells.
- Incubate for 5 hours in a 37°C incubator.
- Carefully add 1 ml of DMEM (with **20**% FCS, w/o antibiotics) to the cells (don't remove the transfection mixture).
- Incubate o/n in a 37°C incubator.

First day after transfection

- The next morning: replace transfection mixture with DMEM complete medium.

Second Day after transfection

- 48 hours after start of transfection harvest the cells by trypsinization.
- Add ~1.7 ml DMEM complete and transfer cells to a 2 ml tube and spin 5 min. at 300×*g*.

For FACS samples:

- Remove supernatant, resuspend cells in PBS.
- Transfer the cells to a FACS tube and spin 5 min. at 300×*g*.
- Remove supernatant and resuspend cells in ~0.5 -1ml PBS.
- Keep cells on ice.
- Perform FACS assay.

For the other samples:

- Remove supernatant as much as possible.
- Continue with the gDNA isolation or store the cell-pellet at -80°C.

Genomic DNA isolation

Isolate the gDNA with the DNeasy Tissue kit. Follow the 'DNeasy Protocol for Cultured Animal Cells'.

- Include RNase A in step 1.
- Elute gDNA in 1×200 μl + 1×100 μl buffer AE in a 2 ml tube.
- Measure concentration of gDNA.

Ordering information

Lipofectamine 2000	Invitrogen	Cat. No.: 11668-027
Opti-MEM I	Invitrogen	Cat. No.: 31985-047
DNeasy Tissue Kit (50)	Qiagen	Cat. No.: 69504
RNAse A solution 100 mg/ml	Qiagen	Mat. No.: 19101

Appendix Figure 3: The Maartje Vogel MCF7 Lipofectamine 2000 Transfection Protocol.

Retrieved from:
http://genome.cshlp.org/content/suppl/2006/10/12/gr.5391806.DC1/LipofectTransfect_gDNAisol_MCF7_060725.pdf

Lipofectamine® 2000 DNA Transfection Reagent Protocol

Transfect cells according to the following chart. Volumes are given on a per-well basis. **Each reaction mix is sufficient for triplicate (96-well), duplicate (24-well), and single well (6-well) transfections, and accounts for pipetting variations.** Adjust the amounts of components according to your tissue culture format. For additional information on scaling your transfection reaction, see page 1.

Timeline		Steps	Procedure Details			
			Component	96-well	24-well	6-well
Day 0	1 	**Seed cells to be 70–90% confluent at transfection**	Adherent cells	1–4 × 10^4	0.5–2 × 10^5	0.25–1 × 10^6
	2 	**Dilute four amounts of Lipofectamine® Reagent in Opti-MEM® Medium**	Opti-MEM® Medium	25 µL × 4	50 µL × 4	150 µL × 4
			Lipofectamine® 2000 Reagent	1, 1.5, 2, 2.5 µL	2, 3, 4, 5 µL	6, 9, 12, 15 µL
	3 	**Dilute DNA in Opti-MEM® Medium**	Opti-MEM® Medium	125 µL	250 µL	700 µL
			DNA (0.5–5 µg/µL)	2.5 µg	5 µg	14 µg
Day 1	4 	**Add diluted DNA to diluted Lipofectamine® 2000 Reagent (1:1 ratio)**	Diluted DNA Total	25 µL	50 µL	150 µL
			Diluted Lipofectamine® 2000 Reagent	25 µL	50 µL	150 µL
	5 	**Incubate**	Incubate for 5 minutes at room temperature.			
			Component	96-well	24-well	6-well
	6 	**Add DNA-lipid complex to cells**	DNA-lipid complex per well	10 µL	50 µL	250 µL
			Final DNA used per well	100 ng	500 ng	2500 ng
			Final Lipofectamine® 2000 Reagent used per well	0.2–0.5 µL	1.0–2.5 µL	5.0–12.5 µL
Day 2–4	7 	**Visualize/analyze transfected cells**	Incubate cells for 1–3 days at 37°C. Then analyze transfected cells.			

Appendix Figure 4: Lipofectamine 2000Transfection Protocol from Life Technologies.

A)

ARC promoter region

ggagcagtgcagactttctctatcgataggtaccgagctcttacgcgtgctagcaagggg
cttggaaccagtccgccgctgcacatccttcttggcttcctggcgccccctatcgggggt
gagcgaagcccccactactaaatggcctctctccactaccccgactatccctgcacataa
actccgttttttttttttctgtttctggtctcttttctctatcgaccagggagcacagga
gttatgggtgggggtgattttgcatttgtagaagcgtccagatactaggtttttaatag
aggaacccctggagggtggaggtgcttggggatccggtgcctagctcccttgcttaggcc
cgggtccagggctggcgcggggccgctgcaatctctgggaagcgggggcttctgggtcgc
gctcagctctgcaatgcatgcgttctgtgatcctggtgagttggtatccttctctgaact
tcctctgtgacaggcagagaagaggggtcgatgacaagatagatggaaagcacctggaaa
ttggcaggccctgagtgggcgctggacgtgtggggccgcgggccgaactgccccctcctg
gcctcatctcctggtgcgccccgggtggggagggaaagctgactacatgcacgtccccag
ccagcgcgcggcctccgcggtgtggctcctagtgcccggggaagaaccctcaccctccct
attcccagcagccctggccgccccgcccccgccgcagctgctgcacccacccccaccctg
cccggccctttctgcaccgtcatctcctgccccgccgaggcttgacccgtgagtggggac
gcccaaaacgggccagaccgggagccccactcccggctgtgggagggaagggaaaccgag
attgggcagaatcagcgccccaccacccgctgcgcgctcggggtcgggggggggcgggggt
ggggtaggactgtggacccgccctgcrcakgamctctcaagctctggtactgggtggagg
gagtaaggrscgggggggaaatcctgcrtcgmactcrgatytgcatctaactarcctgcc
mcatsgtcarcaayggcgacgagctgtcacgrggtgtkccatcctkgaygaactgacktc
tactaacggcmcaagatcaccgtccgcttaggctragcatgcactacgcactgacctaag
gtacctggtacgacgtcaasttgctcgwggtac

B)

ARC promoter region
HindIII restriction site
GFP

tttsgackacgtgagtggggmgcccaaacgggccagaccgggagccccactcccggctgt
gggagggaagggaaaccgagattgggcagaatcagcgccccaccacccgctgcggcgctc
gggggtcgggggggcggggtgggtaggactgtgggccccgccctgccgcaggactctcaa
gctctgggactgggtggagggaggtaaggggcgggggggggaaaatccgtgcagtcgcacc
tcgagatctgcgatctaagtaagcttgccaccatggtgagcaagggcgaggagctgttca
ccggggtggtgcccatcctggtcgagctggacggcgacgtaaacggccacaagttcagcg
tgtccggcgagggcgagggcgatgccacctacggcaagctgaccctgaagttcatctgca
ccaccggcaagctgcccgtgccctggcccaccctcgtgaccaccctgacctacggcgtgc
agtgcttcagccgctaccccgaccacatgaagcagcacgacttcttcaagtccgccatgc
ccgaaggctacgtccaggagcgcaccatcttcttcaaggacgacggcaactacaagaccc
gcgccgaggtgaagttcgagggcgacaccctggtgaaccgcatcgagctgaagggcatcg
acttcaaggaggacggcaacatcctggggcacaagctggagtacaactacaacagccaca
acgtctatatcatggccgacaagcagaagaacggcatcaaggtgaacttcaagatccgcc
gcaacatcgaggacggcggcgtgcagctcgccgaccactaccagcagaacaccccatcg
gcgacggccccgtgctgctgcccgacaaccactacctgagcacccagtccgccctgagca
aagaccccaacgagaagcgcgatcacatggtcctgctggagttcgtgaccgccgcccggg
atcactctcggcatggacgagctgtacaagtaagcggccgcgacgctagagtcggggcgg
ccggccgcttcgagcagacatgatagatacattgatgagttgacaaccacactagatgca
gtgaaaaaatgctttatttgtgaattgtgatgctatggcttaattgtacatatagctgca
taccagtacacacatgcatcgttagttcagttcaagggagtgtgatgagtttttaaggcc
agtaac

Appendix Figure 5: Sequencing results of pGL3-1.005ARC-GFP(luc-). A) Sequenced using the RV3P primer. This only captured the beginning of the 1.005 kB ARC sequence, therefore another primer bound to a different site on the plasmid was used for a second round of sequencing. B) Sequenced using a primer that bound just before the end of the 1.005 kB ARC sequence and captured the end of this sequence and the beginning of GFP.

ARC promoter region

ctcagttactgcmgtctytysttggcttcctggcgccccctatcgggggtgagcgaagcc
cccactactaaatggcctctctccactaccccgactatccctgcacataaactccgtttt
ttttttttctgtttctggtctcttttctctatcgaccagggagcacaggagttatgggtg
gggggtgattttgcatttgtagaagcgtccagatactaggtttttaatagaggaacccct
ggagggtggaggtgcttggggatccggtgcctagctcccttgcttaggcccgggtccagg
gctggcgcggggccgctgcaatctctgggaagcgggggcttctgggtcgcgctcagctct
gcaatgcatgcgttctgtgatcctggtgagttggtatccttctctgaacttcctctgtga
caggcagagaagaggggtcgatgacaagatagatggaaagcacctggaaattggcaggcc
ctgagtgggcgctggacgtgtggggccgcgggccgaactgccccctcctggcctcatctc
ctggtgcgccccgggtggggagggaaagctgactacatgcacgtccccagccagcgcgcg
gcctccgcggtgtggctcctagtgcccgggraagaacmytcaccctccctattcccagca
gccctggcygccccrcccycgccgcagctgctgcacccayscccaccctgcccggcccctt
tctgcmccgtwmtytcctgccccgcwkagsyttgacccgmgagtggrgwacgycaaawsg
ggscagaycgggstccccactccysgctgtggaggkagkgrwaacccasattgwmmrwaa
wcwmcgccccacmcwrswtmckgcsttagrgmwtmg

Appendix Figure 6: Sequencing results of pEGFP-N1-1.005ARC(CMV-). Since there is no RV3P binding site on the pEGFP-N1 plasmid, the primer used to isolate the 1.005 kB ARC sequence from the original pGL3-basic plasmid was used for sequencing. This primer bound to the beginning of the 1.005 kB ARC sequence.