Molecular Mechanism of MicroRNA-203 Mediated Epidermal Stem Cell Differentiation

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Abstract

MicroRNAs (miRNAs) are a family of small, non-coding RNA molecules that play an important regulatory role in many biological processes. However, the precise molecular mechanism of miRNA-mediated regulation is not clear. Here I focused on the role of a deeply conserved miRNA, miR-203, which is one of the most specifically and highly expressed miRNAs in mammalian skin. Previous studies demonstrated that miR-203 is significantly upregulated and, as a result, promotes cell cycle exit during epidermal stem cell differentiation.

To examine the precise dynamics of miR-203-mediated inhibition of self-renewal and dissect the underlying molecular mechanism, I isolated epidermal stem cells from a transgenic miR-203 inducible mouse model. By inducing miR-203’s expression in cultured epidermal stem cells, I demonstrated that miR-203, in a short term, potently inhibits the cell cycle transition between G1 and S phases as early as six hours after induction and, in a long term, greatly reduces the self-renewal capacity of epidermal stem cells. Furthermore, I found that miR-203’s effect is partially, but not completely, reversible – depending on the length of time it is induced in the epidermal stem cells. To examine how miR-203’s targets mediate its function, I showed that individual expression of miR-203 target genes, including Msi2, p63, and Skp2, has distinct phenotypes of rescue in the presence of miR-203. Taken together, these results suggest that miR-203 has a profound impact during epidermal stem cell differentiation and that this regulatory role is mediated by specific target genes with unique cellular functions. These findings provide a novel insight into miRNA-mediated regulation and shed light on the importance of miRNA targeting of multiple target genes with unique cellular functions to regulate broad cellular processes. These observations also raise a possibility about the role of miR-203 and its target genes in skin cancer, as well as other types of epithelial cancers.
Table of Contents:

Abstract.........................................................................................................................1

Table of Contents..........................................................................................................2

Background..................................................................................................................3

Results.........................................................................................................................19

Discussion....................................................................................................................31

Materials and Methods...............................................................................................39

Acknowledgements.......................................................................................................45

References....................................................................................................................46

Supplementary Information..........................................................................................52
Background

MicroRNA

MicroRNAs (miRNAs) are a family of small, non-coding RNA molecules involved in post-transcriptional regulation of gene expression (Bartel, 2004). They are endogenously expressed in a wide spectrum of plants and animals and have regulatory functions in nearly all biological processes.

A brief miRNA history

The first miRNA to be discovered was lin-4 in 1993. While studying the lin-14 gene in C. elegans, Ambros and colleagues found that lin-4 plays a crucial role in development by negatively regulating the level of lin-14 protein (Lee, et al. 1993). They identified two lin-4 transcripts, 22nt and 61nt in length, with complementarity to regions of the lin-14 3’UTR and hypothesized that “lin-4 regulates lin-14 translation via an antisense RNA-RNA interaction” (Lee, et al. 1993.)

It was not until 2000 that a second miRNA was identified, which would lead researchers to realize the conserved, widespread existence of miRNAs. This second miRNA, named let-7, was also discovered in a C. elegans study and was found to have a similar regulatory role to lin-4, as well as complementarity with the 3’UTRs of several genes, including lin-14 (Reinhart, et al. 2000). Later in 2000 the same group published another paper demonstrating let-7 conservation in a wide variety of animal species, including several types of vertebrates as well as invertebrates, suggesting the miRNAs may have important regulatory roles beyond those already understood in C. elegans (Pasquinelli, et al. 2000).

The discovery of these two miRNAs (lin-4 and let-7) prompted the emergence of the field of miRNA research, which has grown significantly in the past decade. MiRNAs are now known
to play important regulatory roles in numerous biological processes, including stem cell self-renewal and differentiation, metabolism, neuronal development, and immune response, to name a few (Yi, et al. 2008; Shah, et al. 2011; Gao, 2010; Wang, et al. 2010). Furthermore, miRNAs have emerged as an important class of regulators that function as tumor suppressors or oncogenes in various types of cancer, including lung cancer, breast cancer, b-cell lymphoma, and leukemia (Kent and Mendell, 2006; Medina and Slack 2008; Calin, et al. 2002; Iorio, et al. 2005; Kumar, et al. 2008). MiRNA dysregulation has been found relevant to many other diseases as well, including Alzheimer’s, cardiovascular disease, Parkinson’s disease, Asthma, Crohn’s Disease, sickle cell disease, and Multiple Sclerosis (Wang, et al. 2008; Small and Olson, 2011; Harraz, et al. 2011; Garbacki, et al. 2011; Brest, et al. 2011; Sankaran, et al. 2011; Tufekci et al 2011).

Understanding miRNA function in normal as well as diseased states not only provides significant insights into important biological processes, but in the future may also be instrumental in disease diagnosis and therapeutics (Trang, et al. 2008).

Function and Biogenesis

MicroRNAs (miRNAs) are a family of small, endogenously expressed non-coding RNA molecules involved in post-transcriptional regulation of gene expression (Bartel, 2004). They regulate the stability and translation of target mRNAs by recruiting the RNA-induced silencing complex (RISC) to their cognate target sites, which are frequently located in target mRNA 3’UTRs (Bartel, 2004). The target recognition is widely believed to be mediated by base-pairing between nucleotides 2-8 of the miRNA, known as the seed sequences, and the mRNA sequences (Bartel, 2009). There are hundreds of known human miRNAs, and their small size and imperfect target recognition allows each one to target many mRNAs (Barbato, et al. 2009). MiRNAs are
predicted to regulate up to 30% of mRNA transcripts, implicating an immense potential regulatory significance (John, et al. 2004).

MiRNA biogenesis is a complex process with many regulatory features, and familiarity with this process is important for understanding miRNA regulation. The canonical miRNA biogenesis pathway is shown in Figure 1. In this pathway, miRNAs are encoded by DNA and are

![Diagram of the canonical miRNA biogenesis pathway](image)

**Figure 1: The canonical miRNA biogenesis pathway.**

Pri-miRNAs are transcribed by polymerase II, then cleaved into pre-miRNAs by Drosha and DGCR8 in the nucleus. Exportin 5 transports pre-miRNAs from the nucleus into the cytoplasm, where they undergo further cleavage by Dicer to form a miRNA duplex. One strand of the duplex gets incorporated into one of four Argonaute proteins and forms the RNA induced silencing complex (RISC). The miRNA, in turn, directs the RISC to its target sites in the 3'UTRs of mRNAs. When a perfect complementarity between the miRNA and the target mRNA occurs, the target is degraded by the cleavage activity of Ago2. In most cases, however, imperfect binding leads to translational repression and destabilization of the targeted mRNA.
transcribed by RNA polymerase II as well as RNA polymerase III (Lee, et al. 2004). In particular, transcription of miRNAs by polymerase II is complexly regulated, giving the cell precise control over when and to what extent miRNAs are expressed. There are two general mechanisms of miRNA transcription, intergenic and intronic. Intergenic miRNAs have their own promoter and regulatory sequences for transcription, similar to mRNAs, and are located in regions of the genome between coding genes. Intronic miRNAs are located within the introns of annotated genes and get co-transcribed along with the mRNA transcript (Ying and Lin, 2006). The initial miRNA transcript is called a primary microRNA (pri-miRNA), and contains miRNAs that form hairpin structures within the transcript. The primary transcript can contain a single miRNA or multiple miRNAs, in which case it is called a polycistronic miRNA cluster. Regardless of the transcription mechanism, the next processing event is performed by Drosha and DGCR8, which cleave the miRNA hairpin(s) out of the surrounding transcript, resulting in a ~65 nucleotide (nt) hairpin intermediate known as a preliminary miRNA (pre-miRNA) (Han, et al. 2004). The pre-miRNA next is transported from the nucleus to the cytoplasm by Exportin-5 (Yi, et al. 2003). Once in the cytoplasm, the pre-miRNA undergoes further processing by Dicer, forming a miRNA duplex (Bernstein, et al. 2001). One strand of this duplex is the mature, approximately 22nt long miRNA, which is loaded into the RNA-induced silencing complex (RISC) and directs it to the target mRNA.

MiRNAs contain a seed sequence approximately 7-8nt long that is responsible for targeting mRNAs. The target mRNA contains a region with complementarity to the miRNA seed sequence, frequently located in its 3’UTR. In many cases, perfect complementarity between the seed sequence and the target mRNA is required for targeting, but complementarity with the rest of the miRNA is not. Once the miRNA has targeted the RISC to the mRNA, the
complementarity of the binding between the miRNA and the target mRNA determines whether the mRNA will be degraded or translationally repressed. If there is perfect complementarity between the entire miRNA and the target mRNA, the RISC will cleave the mRNA, leading to degradation. More often, however, there is imperfect binding between the miRNA and the target mRNA, leading to translational repression (Bartel, 2004; Hammond, 2005).

**Mammalian Skin**

While miRNA regulation has been shown to be relevant in many biological processes and systems, this project focuses on the role of miRNA in regulating proliferation in mammalian skin, focusing on the model organism *Mus musculus*.

**Function and Morphology**

Skin, the largest organ in mammals, provides an important barrier between the body and the outside world. It functions to keep essential components inside the body as well as to keep environmental insults out. The outermost layer of the skin is the epidermis, a stratified epithelium interspersed with hair follicles, sebaceous glands, and sweat glands. The region between these structures is known as the interfollicular epidermis and is comprised of cells called keratinocytes. Morphologically distinct layers of keratinocytes compose the layers of the interfollicular epidermis (Figure 2). Epidermal homeostasis is maintained by the proliferation of a single layer of keratinocytes called the basal layer, where the epidermal stem cells reside. Basal cells undergo asymmetric and symmetric divisions to supply the cells that give rise to the outer layers of the skin, called suprabasal layers. As cells move from the basal layer to the outer layers of the skin, they exit the cell cycle and undergo terminal differentiation. This process occurs throughout the life of the adult, as basal stem cells replenish the outer layers of the skin (reviewed in Blanpain and Fuchs, 2009).
As keratinocytes move from the basal layer to the outer layers of the skin, their gene expression pattern changes. Keratins, the key structural proteins of keratinocytes, are differentially expressed in the basal and suprabasal layers. The basal layer is characterized by expression of keratins K5 and K14, while the suprabasal layers express K1 and K10 (reviewed in Blanpain and Fuchs, 2006). In addition to structural proteins, key regulatory genes are differentially expressed, including transcription factors, cell cycle regulators, and miRNAs.

**Morphogenesis**

During embryonic development, a single layer of basal cells gives rise to the all layers of the interfollicular epidermis as well as the hair follicles. During early development, the skin is comprised of a single layer of progenitor cells (Figure 3). Around embryonic day 13 the single layer of epidermal layers begins to divide asymmetrically to give rise to a second layer. By embryonic day 15 the epidermis consists of two layers, basal and suprabasal and by the adult stage all the layers of the epidermis are present (Yi and Fuchs, 2009). The timing of these stratification events varies depending on the location in the animal, with layers appearing earlier.
in the anterior end of the animal. Precise regulatory pathways control the processes of self-renewal and differentiation as the epidermis develops. One level of control in these processes is mediated by miRNA regulation of gene expression (Yi, et al. 2006).

The miR-203 project

MiR-203 is one of the most highly expressed miRNAs in the epidermis (Yi, et al. 2006), and its expression is normally confined to non-proliferative, differentiated cells, such as suprabasal keratinocytes (Figure 4) (Yi, et al. 2008). This expression pattern, as well as miR-203’s sequence, is conserved in vertebrates, which have a stratified epidermis, but not in lower eukaryotes e.g. C.elegans and Drosophila (Yi, et al. 2008). This conserved pattern suggests a relationship between the evolutionary emergence of a stratified epidermis and the emergence of miR-203 and its suprabasal expression (Yi, et al. 2008). During development, miR-203 expression significantly increases between E13.5 and E15.5, when the suprabasal layer is

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**Figure 3: Morphogenesis of the interfollicular epidermis.**

At embryonic day E13 the epidermis consists of a single layer of basal cells adjacent to the basement membrane. By E15, the basal stem cells give rise to a second, differentiated suprabasal layer. As development continues, the basal cells give rise to the remaining suprabasal layers.
forming (Yi, et al. 2008). Furthermore, miR-203 expression increases when keratinocytes are induced to differentiate in vitro (Yi, et al. 2008). MiR-203’s prevalence, conservation, and expression pattern suggest that it may play a role in regulating proliferation and/or differentiation in mammalian skin.

The purpose of this project is to further elucidate the function of miR-203 in the skin by examining the effects of miR-203 induction on the self-renewal of epidermal stem cells. In addition, this project considers how miR-203 regulates target genes to carry out its regulatory role. One of miR-203’s target genes, p63, has previously been identified, but miRNAs are known to target multiple genes. Thus, to fully understand miR-203’s function, it is necessary to investigate its role in target regulation in depth. This project aims to take miRNA target analysis beyond single target identification and analyze multiple targets on a functional level. Understanding miR-203’s function in the skin will not only shed light on the characteristics of miR-203 and its target genes, but will also provide valuable insight into skin stem cell
proliferation and regulation, which may be relevant to human diseases, such as skin cancer or other types of epithelial cancers.

**Approach to studying miR-203 function and target genes**

This project aims to demonstrate miR-203’s role in regulation of proliferation, as well as to elucidate the role of its target genes by determining which ones are important targets for its function. To accomplish this, we used inducible miR-203 keratinocytes to demonstrate miR-203’s role in regulating proliferation, and followed this with miR-203 target gain-of-function experiments to determine which of its target genes were necessary for its role in regulation. We analyzed short- and long-term proliferation using cell cycle analysis and colony formation assays, respectively.

**Inducible miR-203 transgenic mice**

To study miR-203’s function, we engineered transgenic mice with inducible miR-203 expression using the Tet-on inducible system (Gossen, et al. 1995). These mice contained two transgenes to allow for miR-203 inducible expression (Figure 5).

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**Figure 5: rtTA system for inducible miR-203 expression.**

Transgenic mice containing two constructs were engineered to allow for inducible miR-203 expression using the Tet-on inducible system. **A:** The first construct contained the rtTA gene driven by the K14 promoter so rtTA was constitutively expressed in basal keratinocytes. **B, C:** The second construct contained a tetracycline response element (TRE) and a CMV promoter driving transcription of pri-miR-203 only in the presence of doxycycline (Dox).
The first construct was the reverse tetracycline trans-activator (rtTA) gene, whose expression was driven by the promoter of keratin14 (K14), so rtTA was constitutively expressed in basal keratinocytes. The second construct was the miR-203 gene whose expression was under the control of a promoter regulated by a tetracycline-responsive element (TRE). In the absence of doxycycline, rtTA is unable to bind the TRE so miR-203 is not transcribed. In the presence of doxycycline, however, rtTA binds the TRE, driving transcription of the miR-203 gene. Upon activation, the pri-miR-203 transcript is expressed. This primary transcript then undergoes processing events in the endogenous miRNA biogenesis pathway (Figure 1) and a mature miR-203 is expressed. This inducible system of miR-203 expression allowed us to mimic the induction of miR-203 expression seen during keratinocyte differentiation. Having inducible control over miR-203 expression provided a means to focus specifically on the role of miR-203 in differentiation, without the changing the expression levels of other differentiation factors.

**Cell cycle Analysis**

**Figure 6: The Cell Cycle.**

The cell cycle can be divided into 5 phases: G1, G0, S, G2, and M. G1 occurs after division and is the first growth phase of the cell. G0 is a resting phase that characterizes quiescent cells. During G1 and G0, the cell is diploid and has one copy of each chromosome. S phase is the DNA synthesis phase of the cell cycle, when the chromosomes are in the process of being replicated. G2 is the second growth phase of the cell, following DNA replication. M (Mitosis) is the process of chromosome separation and cell division. During G2 and M, the cell has two copies of each chromosome. Following mitosis, cells enter G1, continuing the cycle through each phase.
One of the key methods employed in these experiments was cell cycle analysis. Analyzing the cell-cycle state of cells gave us a “snapshot” of proliferation in a population of cells. Progression through the cell cycle is a complex, highly regulated process that can be separated into several phases (Figure 6). The rate of progression through the different phases of the cell cycle is an important factor in the short- and long-term proliferative ability of cells. Because one of the key components of the cell cycle is chromosome replication, changes in the amount of DNA can be used to quantify cell cycle progression. In this project, both DNA-staining with Hoechst dye and BrdU incorporation were examined for the analysis of the cell cycle progression and short-term proliferation of cell populations.

**Hoechst:**

A simpler version of cell cycle analysis is based on DNA-staining to measure the amount of DNA content in each cell. We used Hoechst-dye, which quantitatively binds in the minor groove of DNA, to stain the cells after fixation. The amount of Hoechst that a cell incorporates is indicative of the total amount of DNA in

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**Figure 7: Cell cycle profiles.**

A: Left plot shows a cell cycle profile with Hoechst DNA dye. Right plot is the same profile showing G1/G0, S, and G2/M distribution. B: BrdU histogram showing BrdU- and BrdU+ populations. C: BrdU cell cycle profile, showing Hoechst DNA stain plotted against BrdU content. Gates for G1/G0, S, and G2/M are shown.
the cell, so flow cytometry can be used to measure Hoechst fluorescence and give a cell cycle profile indicating the cell-cycle state of each cell and the population as a whole. As seen in Figure 7A, a cell cycle profile will generally have two peaks, where the fluorescence of the second peak is approximately twice the value of the first. The first peak represents cells that are in G1 or G0 and have only one copy of their DNA. The second peak corresponds with twice as much DNA, which represents cells that are in G2/M and have replicated their DNA. The region between the two peaks corresponds with cells that are in S phase and are in the process of replicating their DNA.

BrdU:

A BrdU-based cell cycle analysis can supplement DNA-stain cell cycle analysis to give a more precise cell cycle profile by separating out the cells that are in S-phase. BrdU (Bromodeoxyuridine) is a thymidine analog that gets incorporated into DNA during replication, thereby marking cells that are in S-phase. These cells can then be labeled with a BrdU antibody and analyzed with flow cytometry. A BrdU profile (Figure 7B) will contain two peaks. The BrdU-positive peak corresponds with cells that are in S phase, while the BrdU-negative peak corresponds with all other cells (G0, G1, G2, and M). Plotting a BrdU profile against a DNA-dye profile creates a cell cycle profile with distinct G1/G0, S, and G2/M populations (Figure 7C).

When pulsing with BrdU, only the cells that are in S-phase will incorporate BrdU into their DNA. This means that the population of cells that are BrdU-positive after staining may not represent all of the cycling cells in the population because cells that undergo S-phase in the absence of BrdU do not get labeled. While BrdU is valuable in cell cycle analysis along with a DNA-dye such as Hoechst, it is important to recognize that it labels cells that were in S phase during a specific period of time, and not necessarily all the proliferating cells in a population. In
other words, it gives a “snapshot” of proliferation in a sample of cells. To fully understand the proliferative characteristics of a population of cells it is useful to combine cell cycle analysis with a long-term proliferation assay, such as colony formation.

**Colony Formation**

While we utilized cell cycle analysis in order to obtain a clear illustration of the cell cycle state of cells, we used colony formation to determine the self-renewal ability of cells on a more long-term basis. When cells are seeded at a single cell density and allowed to grow for a long period of time e.g. 10-14 days, they give evidence of different long-term proliferative patterns. First, analyzing the size of each colony over time (number of cells per colony) shows the proliferative rate of individual cells in a population. Analyzing the size of each colony at the end of the experiment shows the comparative long-term proliferation ability of individual cells. Finally, analyzing the number of colonies per well demonstrates the number of cells in a population that have long-term self-renewal ability. Taken together, these analyses can give a clear illustration of long-term proliferation in a population of cells.

Using cell cycle analysis to study the short-term proliferation of a population, as well as colony formation to study the long-term proliferation, we acquire a detailed picture of the proliferative ability of a population of cells. Furthermore, using these techniques we can clearly compare the proliferative ability of multiple cell populations.

**MiR-203 Targets**

One of the main goals of this project was functional analysis of multiple miR-203 targets. This analysis was carried out using gain-of-function experiments (described below). Before these experiments could be carried out, it was necessary to choose miR-203 targets to study. We used a microarray analysis to find genes whose expression decreased when miR-203 expression was
induced in keratinocytes. Then, seed sequence analysis was performed on these genes to determine which ones were likely miR-203 targets. We chose four targets to study, each of which has a unique type of function in the cell: a transcription regulator, a translational regulator, a cell cycle regulator, and a signal transduction regulator. The four targets were p63, Msi2, Skp2, and Vav3.

*p63*

P63 is a homologue of p53, a transcription factor with an essential role in cell cycle regulation (Mills, et al. 1999). P63 is also a transcription factor and it is known to have an important role in regulating proliferation and differentiation in keratinocytes (Truong and Khavari, 2007; Senoo, et al. 2007). P63 expression is limited to the basal layer in the interfollicular epidermis. It is a known target of miR-203, and when miR-203 regulation is inhibited there is an increase of epidermal proliferation and p63 expression (Yi, et al. 2008). For these reasons, p63 was chosen as a target gene to study.

*Msi2*

Msi2 (Musashi homolog 2) is an RNA-binding protein of the Musashi family expressed in neural, germ, and hematopoietic stem cells (Sakakibara, et al. 1996, Siddall, et al. 2006, Kharas et al 2010). Not only has Msi2 been demonstrated as an important regulator of normal stem cell function but its upregulated expression has also been linked to myeloid leukemia (Kharas, et al. 2010, Ito, et al. 2010). Msi2 functions via translational regulation to promote self-renewal of stem cells. Msi2 was chosen as a target to study for two reasons. First, Msi2 seemed to be a likely miR-203 target because of its demonstrated function in stem cells in other systems. Second, its function as an RNA-binding protein involved in translational regulation gave it a unique function among the four target genes chosen.
**Skp2:**

Skp2 (S-phase kinase-associated protein 2) is an F box protein that functions in phosphorylation dependent ubiquitination as a subunit of the ubiquitin protein ligase complex. It ubiquitinates phosphorylated p27, targeting it for degradation (Sutterluty, et al. 1999; Zhu, 2010). Active p27 inhibits activation of cyclins E and D, preventing progression through the restriction point between G1 and S phase. Thus, Skp2 promotes cell cycle progression into S phase by targeting p27 (Assoian and Yung, 2008). Skp2 was chosen as a target gene to study because its role as a cell cycle regulator made it a logical target of miR-203 based on miR-203’s role in regulating proliferation. Furthermore, its function was unique among the four target genes.

**Vav3:**

Vav3 (Vav3 guanine nucleotide exchange factor) is a member of the Vav gene family. Members of this family are guanine nucleotide exchange factors (GEFs) for the Rho family of GTPases, which are involved in signal transduction (Bustelo, 2000). Rho GTPases are involved in signaling pathways for several cellular processes, including cytoskeletal arrangement and activating gene transcription, so Vav3 regulation could potentially have a wide range of effects on the cell (Moores, et al. 2000). For this reason, as well as its unique function compared to the other three target genes, Vav3 was chosen as a miR-203 target gene to study.

**Gain-of-Function**

Gain-of-Function assays were used to determine which of miR-203’s targets are necessary for its ability to regulate proliferation in keratinocytes. To accomplish this, we used constructs containing miR-203 target genes without 3’UTRs. Since miRNAs regulate target gene expression through binding sequences in the 3’UTR of target mRNAs, these constructs produced
mRNAs that were resistant to miR-203 regulation. If certain target genes were necessary for miR-203’s function, expression of this construct would inhibit miR-203’s ability to regulate proliferation, rescuing cells from miR-203’s impact. In this way, gain-of-function miR-203 target constructs were used to determine which of miR-203’s targets’ regulation were necessary for miR-203’s function.

Using short- and long-term proliferation assays within the miR-203 inducible keratinocyte system allowed us to elucidate miR-203’s role in regulating keratinocyte proliferation, as well as gain insights into the role of several of its target genes. Consequently, this project provides novel insights into miRNA-mediated stem cell regulation in mammalian skin.
Results

Induction of miR-203 in inducible keratinocytes

MiR-203 expression is induced with doxycycline in inducible keratinocytes

We used quantitative-PCR to confirm that the inducible keratinocytes expressed miR-203 upon addition of doxycycline (Figure 8A). As early as 6 hours after doxycycline was added, miR-203 expression was induced 3-fold higher than the endogenous level in the noninduced control. MiR-203 expression continued to increase with doxycycline for the first 24-48 hours, with a maximum fold increase of about 8. MiR-205 was used as a control and its expression was not affected with addition of doxycycline. It had been previously shown that miR-203 expression increases 5-15 fold when keratinocytes are induced to differentiate with calcium (Figure 8B: Yi, et al. 2008). Thus, the induction of miR-203 expression achieved in the inducible keratinocytes was within the physiological range of miR-203 induction during differentiation.

Figure 8: Induction of miR-203 expression with doxycycline.
A. qPCR analysis of miR-203 expression in inducible keratinocytes. Cells were cultured in the presence or absence of doxycycline to induce miR-203 expression for 0-48 hours, then RNA was isolated and analyzed with qPCR. P values: *≤0.01, **≤0.001, ***≤0.0001.
B. qPCR analysis of miR-203 expression level during calcium-mediated differentiation. Keratinocytes were cultured in media with low or high calcium concentrations for 24-48 hours to induce differentiation, then RNA was isolated and analyzed with qPCR. From Yi, et al. 2008.
**MiR-203 expression causes an immediate decrease in cell cycle progression**

Once miR-203 induction upon doxycycline addition had been confirmed, we began to investigate its role in regulation of proliferation. Because miR-203 expression coincides with cell cycle exit and differentiation in the skin, we investigated the immediate impact of its expression on the cell cycle. Inducible keratinocytes were cultured in the presence of doxycycline to induce miR-203 expression for 0, 6, 12, 24, or 48 hours and then incubated with BrdU for 1 hour before being collected and fixed for analysis with flow cytometry. As shown in Figure 9B and C, as miR-203 expression increased the percent of cells in S phase decreased while the percent of cells in G1/G0 increased considerably. The percent of cells in G2/M did not change appreciably. These results suggest that miR-203 expression in keratinocytes begins to inhibit progression.

**Figure 9: miR-203 expression causes an immediate decrease in S-phase cells.**

A. BrdU analysis of miR-203’s effect on the cell cycle. Cells were cultured in the presence or absence of doxycycline for 0-48 hours, incubated with BrdU for 1 hour, then collected, fixed, stained with Hoechst, and analyzed with flow cytometry. P values shown are for G1/G0 and S. B. BrdU cell cycle profiles from flow cytometric analysis showing cell cycle phase distribution of cells after 0 or 24 hours of miR-203 induction.
through the cell cycle as early as 6 hours after it is induced and it continues to do so at a higher level as its expression increases with time.

**Figure 10: miR-203 expression inhibits long-term proliferation in inducible keratinocytes.**
Cells were seeded at a single cell density, then doxycycline was added to induce miR-203 expression. Colonies were photographed and counted every 24 hours. **A.** Average number of cells per colony, days 1-5. P values are for induced vs noninduced. **B.** End crystal violet staining of induced and noninduced colonies. **C.** Photographs of representative induced and noninduced colonies for days 3-8.

Induction of miR-203 expression inhibits long-term keratinocyte proliferation

Cell cycle analysis showed that miR-203 expression caused an immediate impact on the cell cycle to inhibit proliferation for at least 48 hours after induction. The next question to be
answered was whether or not miR-203 expression had an impact on long-term proliferation of keratinocytes, which we analyzed with colony formation. Inducible keratinocytes were seeded at a single cell density and allowed to grow in the absence of miR-203 for 24 hours. Then the cells were cultured for 8 more days in the continued presence or absence of doxycycline to induce miR-203 expression. The number of cells per colony was counted every 24 hours for the first five days. At the end of day 8, the colonies were fixed on the plate and then stained with crystal violet for visualization. Figure 10A shows the quantification of the colony size over time based on the number of cells per colony. As expected, noninduced colonies grew at an exponential rate. In contrast, colonies with induced miR-203 expression did not grow. In the presence of miR-203 expression, the total number of colonies that formed was reduced compared to noninduced control (Figure 10B). These results show that miR-203 expression affects both the size and number of colonies that form, indicating that miR-203 affects both the proliferation rate of individual cells as well as the capacity for self-renewal in a population of cells. The cell cycle analysis and colony formation experiments together demonstrate that miR-203 expression inhibits keratinocyte proliferation both by immediate inhibition of progression through the cell cycle as well as long-term inhibition of self-renewal.

**Recovery after miR-203 induction**

When investigating miR-203’s function in regulating proliferation, we were also interested in the amount of time it took cells to respond to miR-203’s presence and their ability to recover in its absence. Using qPCR and cell cycle analysis we saw that as early as 6 hours after induction with doxycycline, miR-203 was being expressed and causing a decrease in S-phase cells (see Figure 9). This suggested that there was a strong response to the presence of miR-203 early after its expression. Furthermore, when miR-203 was continually expressed over
time proliferation continued to be inhibited. We were interested to see if the continued effects on proliferation were due to continuous miR-203 expression or to a one-time change induced by miR-203’s initial expression that would be maintained even in the absence of miR-203.

### Table 1: miR-203 induced for 0 hr 24 hr 48 hr 72 hr Continuous

<table>
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![Figure 1](image)

**Figure 11: Recovery of proliferation after induction of miR-203 is stopped.**
Inducible keratinocytes were seeded at a single cell density, and then miR-203 expression was induced with doxycycline for 24-72 hours. Following induction, doxycycline was removed and the cell were cultured 5-7 more days then stained, counted, and measured to quantify proliferation. A. Chart demonstrating the quantification of colonies based on size. B. Recovery of proliferation based on colony size and number. P values shown are for total # of colonies per well. C. End staining of colonies with crystal violet dye.

**MiR-203’s effects on proliferation are partially reversible**

To investigate this possibility, we used a recovery colony formation assay. Cells were seeded at a single cell density and allowed to grow in the absence of miR-203 for 48 hours. At 48 hours, miR-203 expression was induced with doxycycline. Cells were then cultured with
doxycycline-free media beginning at various time points and allowed to grow for several more days in the absence of miR-203 induction. At the end, the colonies were fixed, stained, and then quantified according to size. As seen in Figure 11, there was a steady decrease in the number of colonies of all sizes as they were exposed to doxycycline for longer amounts of time. Colonies that were exposed to miR-203 for longer amounts of time had a stronger non-proliferative response. Cells exposed to doxycycline for 24 hours and then allowed to recover for 7 days were able to form large colonies, but they weren’t able to form as many as the non-induced controls. At the other end of the spectrum, cells exposed for 72 hours were unable to form large colonies and had few small colonies and were comparable to those exposed to continuous miR-203 induction. qPCR performed at the end of the recovery experiment showed that miR-203 was not present in cells after they were given time to recover (supplementary figure 3). These data suggest that the first 72 hours of miR-203’s expression cause a strong but partially reversible response in long-term proliferative ability of keratinocytes.

**Rescue: miR-203 target gain-of-function**

**Target verification**

Four miR-203 targets were chosen to study to further elucidate miR-203’s function in regulating keratinocyte proliferation. Potential target genes were chosen based on microarray and seed sequence analysis. From the list of potential target genes, four genes with unique functions in the cell were chosen to study. QPCR was used to verify that the targets were regulated by miR-203 within the miR-203 inducible expression system. RNA isolated from keratinocytes with induced miR-203 expression showed decreased expression of the four target genes (Figure 12). These data are consistent with the knowledge that miR-203 functions by downregulating target gene expression.
Rescue of cell cycle progression by Skp2

Cell cycle rescue assays were used to determine which target genes of miR-203 were necessary targets for downregulation in order to achieve its effect on the cell cycle. Inducible keratinocytes were infected with MIGR constructs for p63, Msi2, Skp2, Vav3, or empty vector control. Each MIGR construct contains a miR-203 target gene without a 3’UTR so that it can’t be downregulated by miR-203. If one of these genes is a necessary target of miR-203, then when miR-203 expression is induced in keratinocytes there will be a decreased effect on the cell cycle. As shown in Figure 13A, when miR-203 expression is induced in cells infected with the MIGR Msi2, p63, or Vav3 constructs there is a similar decrease in S-phase cells as seen with the vector control. When miR-203 expression is induced in cells infected with the MIGR Skp2 construct, on the other hand, the decrease in S phase cells is approximately 2-fold less severe. These results suggest that while Msi2, p63, and Vav3 are not necessary target genes of miR-203 in order for it to be able to affect the cell cycle in keratinocytes, targeting of Skp2 is essential.
Rescue of colony formation by p63, Msi2, Skp2, and Vav3

Colony formation assays were used to determine which target genes of miR-203 were necessary targets for downregulation in order to inhibit long term proliferation. Inducible keratinocytes were infected with MIGR constructs for p63, Msi2, Skp2, Vav3, or empty vector control, then sorted with Fluorescence-Activated-Cell-Sorting (FACS) using GFP as a marker for infected cells. Infected cells were then seeded at a single cell density (1000 cells per well) and

allowed to grow for 24 hours. After 24 hours, doxycycline was added daily. Colonies were marked and tracked daily as described in the methods section. Figure 13B shows the data from counting the number of cells per colony per day. The noninduced cells grew at an exponential rate for all the constructs and the noninfected control, as expected. The noninfected and empty vector control infected cells did not grow when miR-203 expression was induced with doxycycline, also as expected. Cells expressing the MIGR Vav3 construct or the MIGR Skp2 construct behaved similar to the vector infected cells in daily proliferation rates. MIGR Msi2 and MIGR p63 infected cells, on the other hand, had an intermediate rate of proliferation. When considering the final colony size and number (Figure 13 C and D), the noninfected and vector controls had many colonies in the absence of miR-203, as did all noninduced populations. In the presence of miR-203 induction, the noninfected and vector controls did not grow, as expected. In the presence of miR-203, the MIGR Msi2 infected cells were able to form an intermediate number of small colonies as well as a few large colonies. The MIGR Skp2 and MIGR p63 infected cells were able to form small colonies and an occasional large colony in the presence of miR-203. MIGR Vav3 infected cells were able to form a very small number of small colonies when miR-203 expression was induced. These results show that expressing Msi2, p63, and Skp2 individually and eliminating miR-203’s ability to target them results in partial rescue of keratinocytes from the long term inhibition of proliferation induced by miR-203 expression. Taken along with the cell cycle rescue results, this suggests that Skp2, Msi2, and p63 are all necessary targets of miR-203 for its role in regulating keratinocyte proliferation.

**Combinatorial Rescue**

After finding that Skp2, Msi2, and p63 were all able to partially rescue keratinocytes from miR-203’s effects on the cell cycle and long-term proliferation, we were interested in the
combinatorial effects of expressing these target genes in a rescue assay. These three genes are involved in different cellular processes relating to proliferation and self-renewal, and miRNAs are known to function by downregulating several target genes. If miR-203 combinatorially targets these genes in order to affect proliferation on several levels, then expressing multiple MIGR constructs in keratinocytes may be able to more fully rescue them from miR-203’s effects on proliferation.

Rescue of cell cycle progression does not increase with combinatorial miR-203 target expression

To examine the possibility of enhanced rescue with combinatorial target expression, we infected keratinocytes with multiple MIGR constructs and carried out cell cycle analysis and colony formation assays as before. As shown in Figure 14, the percent of cells in S phase when miR-203 is induced was similar between the vector control, Msi2, p63, and Vav3 infected cells. There was an increase in S phase when induced cells expressed the Skp2 construct. These data correlate with the findings of the earlier single construct rescue experiments. The cells infected with combinations of p63/Skp2 (ps), and Msi2/p63/Skp2 (mps) had a similar percentage of cells in S phase compared to cells expressing Skp2 alone. The Msi2/Skp2 (ms) combination had an intermediate level of proliferation and the Msi2/p63 (mp) combination was similar to the vector control. None of the combinations resulted in a greater cell cycle rescue than when infected with Skp2 alone. These results suggest that miR-203 targets Skp2 to cause the decrease in S-phase cells, and it does not rely on combinatorial targeting of Msi2, p63, and Skp2 together.

Colony formation rescue is not enhanced with combinatorial miR-203 target expression

To determine whether or not combinatorial expression of miR-203 target genes led to an increased rescue from miR-203’s effect on self-renewal, we used colony formation assays. First, we infected inducible keratinocytes with individual or multiple MIGR constructs. Then, we
sorted cells with FACS using GFP fluorescence to isolate infected cells. GFP positive cells were seeded at a single cell density (1000 cells per well) and allowed to grow for 10 days in the presence or absence of doxycycline. Similar to the previous colony formation assay with individual target genes, we saw partial rescue of long-term proliferation by Msi2, p63, and Skp2 (Figure 14B, C). When these three targets were combined, the partial rescue of long-term
proliferation was similar to the rescue seen Msi2 and p63 individually, with no apparent increase. These results indicate that miR-203 does not rely on combinatorial targeting of Msi2, p63, and Skp2 in order to carry out its inhibitory effect on long-term proliferation.
Discussion

The purpose of this project is twofold; first, this project aimed to elucidate the function of miR-203 in the skin by examining the effects of its overexpression on keratinocyte proliferation. Second, this project endeavored to gain a further understanding of miR-203’s function by analyzing multiple miR-203 target genes on a functional level.

MiR-203 inhibits keratinocyte proliferation

Using keratinocytes isolated from transgenic mice with inducible miR-203 expression, we found that miR-203 overexpression inhibited cell proliferation. MiR-203 overexpression had an immediate impact on the cell cycle, causing a decrease in S-phase cells as early as 6 hours after induction. This effect increased as miR-203 expression increased, up to a 75% decrease in S-phase cells when miR-203 expression increased ~8-fold. Furthermore, miR-203 induction was also found to have a strong inhibitory effect on long-term proliferation in keratinocytes. Taken together, these data are evidence that miR-203 plays a role in regulating keratinocyte proliferation. The previous knowledge that miR-203 expression is limited to suprabasal cells in the interfollicular epidermis, as well as its inhibitory effect of proliferation demonstrated in this project, suggest that it plays a role in inducing cell-cycle exit as basal layer stem cells divide and give rise to non-cycling, differentiated suprabasal cells.

When examining miR-203’s function in inhibiting keratinocyte proliferation, we studied the reversibility of its effects on long-term proliferation. When miR-203 expression was induced for 24-72 hours and then the induced cells were allowed to grow in its absence, there was a partial, but not complete, recovery from miR-203’s effects. This incomplete reversibility suggests that miR-203 expression is necessary for the transition from basal to suprabasal cell, inducing cell cycle exit and differentiation. Thus, the first 24-48 hours of miR-203 expression
promote a rapid transition, but expression beyond that time frame may not be as essential for defining the non-cycling, differentiating characteristics of suprabasal cells. However, it is known that miR-203 expression is frequently downregulated in cancer cells. Because miR-203’s inhibitory effect is partially reversible for the first 24-48 hours, downregulation of miR-203 may be an important step in inducing cancer in cells that express miR-203, such as the suprabasal layers of the skin that can give rise to squamous cell carcinoma. For this reason, the continual expression and incomplete reversibility of miR-203’s inhibitory effect may be part of a tumor-suppressor mechanism, so that downregulation of miR-203 is not sufficient to induce cancer in cells that have been expressing miR-203 for longer amounts of time.

**MiR-203 targets several unique genes to accomplish its regulatory role**

While miRNAs are known to function through regulation of numerous target genes, before this project, there was only one verified miR-203 target, p63. Once miR-203’s role in regulating proliferation was clear, the second aim of this project was to analyze multiple potential miR-203 targets on a functional level. Four target genes with unique functions were chosen based down-regulation in the presence of miR-203 and 3’ UTR miR-203 seed sequences: Msi2, p63, Skp2, and Vav3. We analyzed the functional role of these genes in miR-203 regulation using constructs to express these genes without their 3’UTR regions so they could not be targeted by miR-203. Skp2 was found to partially rescue keratinocytes from miR-203’s immediate impact on the cell cycle, decreasing miR-203’s inhibitory role by ~50%. Furthermore, Msi2, p63, and Skp2 were individually able to partially rescue keratinocytes from miR-203’s effects on long-term proliferation. These results suggest that miR-203’s regulatory role in keratinocytes is mediated by multiple targets with unique functions. This is consistent with the idea that miRNAs are part an important regulatory mechanism that allows the cell to
control processes on a broad scale by regulating the expression of multiple genes, while only having to activate or inhibit transcription of a single factor.

The partial rescue of keratinocytes from miR-203’s impact on proliferation by individual target genes raised the question of whether the combinatorial expression of the target genes would provide a more complete rescue. The three genes that were found to partially rescue keratinocyte proliferation from miR-203 regulation, Msi2, p63, and Skp2, were examined in combination for their effects on cell cycle progression and long-term proliferation. No combination of the three target genes showed an effect on the cell cycle progression greater than the individual effect of Skp2, suggesting that miR-203 does not rely on combinatorial targeting of these genes to inhibit cell cycle progression. Furthermore, combining the three target genes in colony formation experiments did not result in a greater rescue effect than that seen with individual targets, suggesting that miR-203 does not rely on combinatorial targeting of Msi2, p63, and Skp2 to inhibit long-term proliferation in keratinocytes. These results do not rule out the possibility of combinatorial rescue, however. It is possible that combining other miR-203 target genes than the ones used in this project could lead to a more complete rescue from miR-203’s inhibitory effect on short- and long-term proliferation. One caveat to the combinatorial rescue experiment performed in this project is that all of the rescue constructs express GFP. Therefore, although it was possible to select for cells that expressed at least one construct, it was impossible to know which constructs were expressed in a GFP cell expressed based on fluorescence alone. Thus, analyzing cell cycle progression with flow cytometry gave an idea of the cell cycle state of cells expressing rescue constructs, but was not sufficient to separate cells that were singly infected from those expressing multiple rescue constructs. In addition, using FACS to select GFP positive cells to seed in colony formation experiments ensured that each
colony represented expression of at least one target gene. However, FACS sorting could not
discriminate between cells that expressed individual or multiple target gene constructs, so the
colonies studied may not have all been expressing all three target genes. To draw more clear
conclusions about the combinatorial rescue ability of miR-203’s target genes, it would be useful
to create constructs that could be used to select for multiple-construct expression, for example
constructs expressing different fluorescent proteins. Based on the results from this project,
however, it appears that combinatorial expression of miR-203 target genes does not lead to a
greater rescue from inhibition of cell cycle progression or long-term proliferation and self-
renewal.

**Proposed Model of miR-203 Function in the Skin**

The results in this project demonstrated that miR-203 inhibits keratinocyte proliferation
through downregulation of specific target genes with unique functions. Based on these results, as
well as previous knowledge of miR-203 and the target genes studied, we propose the following
model for miR-203’s role in regulating epidermal homeostasis (Figure 15). Basal stem cells are
characterized by cell cycle progression, self-renewal and proliferation, and a lack of
differentiation. These characteristics are maintained by expression of many genes with unique
functions. A few of these genes include Skp2, Msi2, and p63. Skp2 promotes cell cycle
progression by promoting transition from G1/G0 into S-phase. Msi2 is a translational regulator
that promotes self-renewal of the basal stem cells. P63 promotes proliferation and inhibits
differentiation through transcriptional regulation. Expression of many genes that function in
diverse biological processes allows basal cells to maintain their stem-cell characteristics.

When basal cells undergo asymmetric division to give rise to suprabasal cells, they
transition to become non-proliferative cells on a pathway of terminal differentiation. miR-203
expression dramatically increases during this transition and continues through the life of suprabasal cells as they travel away from the basal layer and undergo further differentiation. The first 24-48 hours of miR-203 expression is essential, as it downregulates target genes to cause rapid changes in the cell to give it suprabasal characteristics. By targeting Msi2, Skp2, and p63, miR-203 is able to inhibit cell cycle progression and proliferation, while promoting
differentiation. In this way, several cellular processes are regulated by expression of a single regulatory factor, insuring a rapid, dramatic transition from basal to suprabasal cell.

**Larger Implications**

**Understanding stem cell function**

Understanding miR-203’s function in the skin not only sheds light on the characteristics of miR-203 and its target genes, but also gives valuable insight into stem cell regulation in the skin. The two defining characteristics of stem cells are their capacity for self-renewal and their potential to give rise to differentiated daughter cells. Several of miR-203’s characteristics suggest that it plays a key role in the transition from basal stem cell to differentiated suprabasal cell in the epidermis. MiR-203 expression correlates with differentiation *in vivo* both during development as the suprabasal layers begin to form, as well as in the adult where its expression is restricted to the suprabasal layers of the epidermis. MiR-203 expression is induced in cultured keratinocytes during calcium-mediated differentiation, demonstrating that the relationship between miR-203 and differentiation also exists *in vitro*. Considering miR-203’s importance in differentiation, as well as its role in regulating proliferation demonstrated in this project, it is apparent that elucidating the functions miR-203 and its targets goes hand in hand with understanding stem cell regulation in the skin. Understanding stem cell regulation in the skin could provide important insights into skin wound healing and regeneration to aid in treatments such as skin grafts.

**Understanding regulation of proliferation in the skin**

Because miR-203 functions in regulation of proliferation and differentiation, it is an interesting target to study in relation to cancer. MiR-203’s inhibition of stem cell proliferation designates it as a potential tumor suppressor. In fact, miR-203 has been shown to suppress cancer
progression and its downregulation has been connected to several types of cancer (Bo, et al. 2011; Saini, et al. 2010; Castilla, et al. 2010; Chiang, et al. 2011, Wijnhoven, et al. 2010; Furuta, et al. 2010; Bueno, et al. 2008; Mathé, et al. 2009; Viticchie, et al. 2011). MiR-203 inhibits proliferation by regulating target genes, so this project not only sheds light on miR-203 as a potential tumor suppressor, but also on the mechanism by which it acts and the potential oncogenic effects of its target genes. Because miR-203 is an inhibitor, its role as a potential tumor suppressor implies that those genes which it inhibits are potential oncogenes. Studying the effects of miR-203 target gene gain-of-function on different aspects of proliferation also gives insight into their roles a potential oncogenes, as Skp2, Msi2, and p63 were all shown to promote short- or long-term proliferation. A further understanding miR-203 and its target genes could provide valuable insights into skin cancer as well as other types of epithelial cancers.

**Future directions**

The results found in this project present the opportunity for a wide variety of future studies. First, while this project investigated the necessity of certain miR-203 target genes, it did not address their sufficiency. So, in addition to the gain-of-function rescue assays using miR-203’s targets, a more complete picture could be gained if loss-of-function assays were performed with the same target genes. If targeting of specific target genes is sufficient to induce the effects on proliferation caused by miR-203 expression, this study could provide insight into which of miR-203’s targets are causing its effect on proliferation. Thus, studying the effects of knocking down miR-203 target genes individually and in combination could further elucidate the mechanism behind how miR-203 exhibits its effect on keratinocyte proliferation. Also, an analysis of more miR-203 targets may give a wider view of how it carries out its effects in the cell. Although the genes studied in this project were not shown to have a combinatorial effect on
rescue of proliferation, we know that miRNAs function by targeting multiple genes at once. So, studying a wider range of genes could provide insight into which combinations of genes are being targeted by miR-203 to inhibit cell cycle progression and long term proliferation. In addition to studying more of the molecular mechanisms related to miR-203’s function, it would also be of value to do an in vivo analysis of miR-203 overexpression to analyze its physiological effects. The environment of the skin contains many factors that are not mimicked in cell culture, such as signaling factors and stratification of epidermal layers. Studying miR-203’s function in an in vivo model, therefore, could show how the effects demonstrated in this project are physiologically relevant.

In addition to understanding miR-203’s normal function in the skin, it would also be of value to study its role in cancer. MiR-203’s role in inhibiting proliferation, as well as its downregulation in certain cancers suggests it may act as a tumor suppressor. Studying this possibility, as well as the potential oncogenic roles of miR-203’s target genes, could give valuable insights into the molecular mechanisms involved in skin cancer or other types of cancer involving dysregulation of miR-203 and/or its target genes.
Materials and Methods

RNA isolation

Cells were washed with 100% PBS, then incubated with TRIzol reagent for 10min at room temperature. Next, chloroform (equal by volume to 20% of the amount of TRIzol used) was added for an additional 2-3 minutes at room temperature. This solution was centrifuged for 14 min at 14,000 rpm at 4°C. The top layer of the resulting supernatant (containing RNA) was transferred to a new 1.5mL low retention tube and mixed with an equal amount of isopropanol, then incubated at room temperature and centrifuged as before. The supernatant was removed and the pellet (containing RNA) was washed with 75% EtOH, then centrifuged for 5 min at 8000rpm at 4°C. The EtOH was removed and the pellet was incubated at 37°C for 5-7 min to evaporate off any remaining EtOH. The RNA was resuspended in RNase free water and stored at -80°C.

Quantitative PCR

Real-time PCR quantification was performed using the Qiagen miScript system following the manufacturer’s protocol. For miRNA, 40 cycles of 95°C for 15s, 55°C for 30s, and 72°C for 30s were run and Sno25 was used as the internal control. For mRNA, 40 cycles of 95°C for 10s, 60°C for 10s, and 72°C for 30s were run and GAPDH and HPRT were used for the internal controls.

Statistical Analysis

P values were calculated using an unpaired t test using the online calculator from GraphPad software. Statistically significant values were labeled as follows: *:≤0.01, **:≤0.001, and ***:≤0.0001. Values ≥0.05 were deemed not statistically significant. A P-value reference chart can be found in the supplementary information. Error bars designate standard deviation.
Cell Culture

Keratinocyte Cell Culture

Keratinocytes were cultured using E low calcium media (0.05 mM calcium chloride) and were split using Trypsin (incubation at 37°C for ~10min) when they reached ~90% confluence.

Isolation of keratinocytes for cell culture

Back skin was harvested from newborn (P1-P2.5) mice and incubated overnight in Dispase at 4°C. Then the epidermis was separated from the dermal layer, incubated in Trypsin for 10-12 min at 37°C, filtered through at 70um filter, centrifuged (5min at 0.3rcf at room temperature), and resuspended in E low calcium media on feeder cells. Keratinocytes were cultured on feeder cells for the first two passages.

Inducible keratinocytes

Inducible keratinocytes were isolated from k14rtTA/pTRE2miR-203 transgenic mice. These mice were created by mating k14rtTA mice with pTRE2miR-203 mice. The k14rtTA construct is a k14 promoter driving the expression of the reverse tetracycline trans-activator (rtTA) gene. The pTRE2miR-203 construct is a tetracycline response element (pTRE2) followed by a CMV2 promoter driving expression of the pri-miR-203 gene.

293 cell culture

BOCS and 293FT cells were cultured using Dulbecco’s Modified Eagle Medium (DMEM) containing 10% FBS, 1% Penicillin/Streptomycin, and 1% Sodium Pyruvate. They were split using Trypsin (incubation at 37°C for ~5min) when they reached ~90% confluence.

Storing cells

Aliquots of Keratinocytes and 293 cells were stored in liquid nitrogen or at -80°C in 95% media with 5% DMSO.
**Induction with doxycycline**

MiR-203 expression was induced by adding doxycycline to the media at a concentration of 3ug of doxycycline per 1mL of media. For induction for longer than 24 hours, fresh doxycycline was added daily. See supplementary information for a more detailed experimental timeline.

**Flow Cytometry**

**Cell cycle analysis sample preparation**

Reagents from the BD Pharmingen BrdU kit were used in the following procedure. 30-60 min before cells were harvested, they were incubated with BrdU at a concentration of 3.33ug/mL. At the end of the BrdU treatment, cells were washed with PBS and trypsinized (~10min at 37°C). Trypsinization was quenched using E low calcium media. Then cells were centrifuged (5min, 0.3rcf, room temp) and the supernatant was discarded. The cells were resuspended in 100uL BD Cytofix/Cytoperm Buffer and incubated at room temp for 15 min. Cells were washed by adding 1mL 1x BD Perm/Wash Buffer, then centrifuged as before and the supernatant was removed. Cells were resuspended in 1mL PBS then stored overnight at 4°C. The following day steps 3-6 of the BD Pharmingen BrdU Flow Staining protocol were followed with the following adaptations: The anti-BrdU antibody was diluted 200x rather than 50x and the wash step following antibody labeling was carried out with 2mL 1x BD Perm/Wash buffer rather than 1mL. Cells were then incubated at 37°C for 30 min in 500uL PBS with 1x Hoechst dye (33342), then incubated overnight at 4°C. Flow cytometric analysis was carried out the following day.
Cell sorting sample preparation

Cells were washed with PBS and trypsinized for ~10 min, then centrifuged and resuspended in sorting media (PBS, 1x Hoechst, 2% Serum). Cells were kept on ice, sorted using FACS, and reseeded.

Gating and analysis: BrdU cell cycle analysis

All samples were first gated using forward scatter/side scatter plots to separate cells from debris, then gated with forward scatter/pulse width plots to select for single cells. Next, samples were gated for GFP fluorescence (when applicable). Finally, Hoechst fluorescence and BrdU fluorescence were plotted against each other and G1/G0, S, and G2/M phase gates were created to quantify the cell cycle phase of the cells in each sample (see cell cycle analysis section in introduction/background for more information). See supplementary information for example gating profiles.

Gating and analysis: cell sorting

All samples were first gated using forward scatter/side scatter plots to separate cells from debris, then gated with forward scatter/pulse width plots to select for single cells. Next, samples were gated for viability based on the presence or absence of Hoechst dye (live cells are Hoechst-negative). Finally, cells were gated for GFP fluorescence and sorted accordingly. See supplementary information for example gating profiles.

Colony formation assays

Keratinocytes were seeded at a single cell density (1000 cells/well of a 6 well plate, unless otherwise specified) and allowed to grow for 8-10 days. To count number of cells per colony, 6 single cells per well were marked 24 hours after seeding by circling their location on the bottom of the plate. Every subsequent 24 hours, photographs were taken of each marked
colony and at the end of the experiment the number of cells in each colony was counted. For colonies with greater than 500 cells, the colony was divided into fourths and the average number of cells in two quarters was multiplied by 4 to approximate the total number of cells in the colony. At the end of the experiment, cells were washed with PBS, fixed on the plate with 4% PFA for 10 min at room temperature, stained with 0.05% Crystal Violet dye for 30 min, and finally washed twice with water. To quantify the final colony size, all visible colonies in each well were measured and counted based on size. To quantify the final colony number, all visible colonies in each well were counted. See supplementary information for a more detailed experimental timeline.

**Rescue assay**

**MIGR constructs**

The rescue experiments were conducted using retroviral MIGR constructs containing miR-203 targets (Msi2, p63, Skp2, and Vav3) without their 3’UTRs, as well as an empty MIGR vector control. The MIGR vector contains GFP expression driven by an IRES (internal ribosomal entry site). This construct allows for expression of GFP as well as the gene of interest, both driven by the gene of interest’s promoter. This way, GFP expression can be used to confirm expression of the gene of interest.

**Transfection**

The MIGR construct was transfected into BOCS along with the PCL packaging plasmid using PEI transfection. 7ug MIGR, 3ug pCL, and 70ug PEI were used to transfect 10cm dishes. The plasmid/PEI mix was incubated at room temperature with 1mL serum free DMEM media for 20 min and then added to BOCS cells at ~80% confluence. 12-24 hours later, the media was
changed to E low calcium to make virus for infecting keratinocytes. 24 and 48 hours later, viral supernatant was collected and used for infection or frozen in liquid nitrogen and stored at -80°C.

Infection

MIGR viral supernatant was filtered through a 0.45um filter and then polybrene was added at a concentration of 8μg/mL. Keratinocyte media was removed and replaced with the virus, then plates of infected cells were centrifuged at 1500 rpm for 45 min at room temp. 3-6 hours later, the virus was removed, cells were washed with PBS and given new E low calcium media. For combinatorial rescue experiments, virus for each construct was made separately and then multiple viruses were added to keratinocytes using the same procedure as described above.

Analysis

Cell cycle and colony formation experiments were performed as described above. Noninfected as well as MIGR vector infected cells were used as controls in all experiments. See supplementary information for detailed timelines of cell cycle rescue and colony formation rescue experiments.
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Finally, I would like to acknowledge Victoria Hildreth and the Biological Sciences Initiative for providing me with the initial opportunity to work in the lab.
References


signaling in antiviral innate immunity by targeting suppressor of cytokine signaling 1."


Supplementary Information

**Experiment Timelines:**

**Figure 8A: miR-203 expression in inducible keratinocytes**

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<th>Time</th>
<th>Activity</th>
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<tr>
<td>1</td>
<td>0hr</td>
<td>seed cells for experiment</td>
</tr>
<tr>
<td>2</td>
<td>24hr</td>
<td>add dox to 48hr sample</td>
</tr>
<tr>
<td>3</td>
<td>48hr</td>
<td>change media on all samples, add dox to 24hr and 48hr samples</td>
</tr>
<tr>
<td>4</td>
<td>66hr</td>
<td>add dox to 6hr sample</td>
</tr>
<tr>
<td>4</td>
<td>72hr</td>
<td>collect cells in trizol for RNA analysis</td>
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**Figure 9: Impact of miR-203 expression on the cell cycle**

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<td>24hr</td>
<td>add dox to 48hr sample</td>
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<tr>
<td>3</td>
<td>48hr</td>
<td>change media on all samples, add dox to 24hr and 48hr samples</td>
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<tr>
<td>3</td>
<td>60hr</td>
<td>add dox to 12hr sample</td>
</tr>
<tr>
<td>4</td>
<td>66hr</td>
<td>add dox to 6hr sample</td>
</tr>
<tr>
<td>4</td>
<td>72hr</td>
<td>incubate with BrdU 60min, collect/fix cells</td>
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<tr>
<td>5</td>
<td></td>
<td>prep for flow cytometry</td>
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<tr>
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**Figure 10: Impact of miR-203 expression on colony formation**

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<td>seed cells at single cell density</td>
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<tr>
<td>2</td>
<td>24hr</td>
<td>add dox to induced samples</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mark colonies to track</td>
</tr>
<tr>
<td></td>
<td></td>
<td>take pictures of tracked colonies</td>
</tr>
<tr>
<td>3-10</td>
<td>48-240hr</td>
<td>add dox to induced samples</td>
</tr>
<tr>
<td></td>
<td></td>
<td>take pictures of tracked colonies</td>
</tr>
<tr>
<td>10</td>
<td>240hr</td>
<td>fix colonies with PFA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>stain colonies with crystal violet dye</td>
</tr>
</tbody>
</table>

**Figure 11: Recovery**

<table>
<thead>
<tr>
<th>Day</th>
<th>Time</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0hr</td>
<td>seed cells at single cell density</td>
</tr>
<tr>
<td>2</td>
<td>24hr</td>
<td>add dox to 24hr, 48hr, 72hr, continuous samples</td>
</tr>
<tr>
<td>3</td>
<td>48hr</td>
<td>change media to no dox for 24hr sample</td>
</tr>
<tr>
<td></td>
<td></td>
<td>add dox to 48hr, 72hr, continuous samples</td>
</tr>
<tr>
<td>4</td>
<td>72hr</td>
<td>change media to no dox for 48hr sample</td>
</tr>
<tr>
<td></td>
<td></td>
<td>add dox to 72hr, continuous sample</td>
</tr>
<tr>
<td>5</td>
<td>96hr</td>
<td>change media to no dox for 72hr sample</td>
</tr>
<tr>
<td></td>
<td></td>
<td>add dox to continuous sample</td>
</tr>
<tr>
<td>6-10</td>
<td>120-240hr</td>
<td>add dox to continuous sample</td>
</tr>
<tr>
<td>10</td>
<td>240hr</td>
<td>fix colonies with PFA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>stain colonies with crystal violet dye</td>
</tr>
</tbody>
</table>
### Figure 12: miR-203 target gene expression

<table>
<thead>
<tr>
<th>Day</th>
<th>Time</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0hr</td>
<td>seed cells for experiment</td>
</tr>
<tr>
<td>2</td>
<td>24hr</td>
<td>add dox to 24hr induced sample</td>
</tr>
<tr>
<td>3</td>
<td>48hr</td>
<td>collect samples in trizol for RNA analysis</td>
</tr>
</tbody>
</table>

### Figure 13A/14A: Cell cycle rescue

<table>
<thead>
<tr>
<th>Day</th>
<th>Time</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0hr</td>
<td>seed BOCS cells</td>
</tr>
<tr>
<td>2</td>
<td>24hr</td>
<td>transfect BOCS cells</td>
</tr>
<tr>
<td>3</td>
<td>36hr</td>
<td>change media on transfected cells to E low calcium</td>
</tr>
<tr>
<td></td>
<td>48hr</td>
<td>seed inducible keratinocytes for experiment</td>
</tr>
<tr>
<td>4</td>
<td>60hr</td>
<td>infect inducible keratinocytes with virus from BOCS cells</td>
</tr>
<tr>
<td>4</td>
<td>72hr</td>
<td>add dox to induced samples</td>
</tr>
<tr>
<td>5</td>
<td>96hr</td>
<td>change media (+/-dox) on all samples)</td>
</tr>
<tr>
<td>6</td>
<td>120hr</td>
<td>incubate with BrdU, collect/fix cells</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>prep for flow cytometry</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>flow cytometry analysis</td>
</tr>
</tbody>
</table>

### Figure 13B-D/14B-C: Colony Formation Rescue

<table>
<thead>
<tr>
<th>Day</th>
<th>Time</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0hr</td>
<td>seed BOCS cells</td>
</tr>
<tr>
<td>2</td>
<td>24hr</td>
<td>transfect BOCS cells</td>
</tr>
<tr>
<td>3</td>
<td>36hr</td>
<td>change media on transfected cells to E low calcium</td>
</tr>
<tr>
<td></td>
<td>48hr</td>
<td>seed inducible keratinocytes for experiment</td>
</tr>
<tr>
<td>4</td>
<td>60hr</td>
<td>infect inducible keratinocytes with virus from BOCS cells</td>
</tr>
<tr>
<td>5</td>
<td>84hr</td>
<td>change media on infected cells</td>
</tr>
<tr>
<td>6</td>
<td>108hr</td>
<td>sort using FACS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>seed sorted cells at low density</td>
</tr>
<tr>
<td>7</td>
<td>132hr</td>
<td>add dox to induced samples</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mark colonies to track</td>
</tr>
<tr>
<td></td>
<td></td>
<td>take pictures of tracked colonies</td>
</tr>
<tr>
<td>8-15</td>
<td>156hr-372hr</td>
<td>add dox to induced samples</td>
</tr>
<tr>
<td></td>
<td></td>
<td>take pictures of tracked colonies</td>
</tr>
<tr>
<td>15</td>
<td>372hr</td>
<td>fix colonies with PFA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>stain colonies with crystal violet dye</td>
</tr>
</tbody>
</table>
P-value Reference Charts:

**Figure 8A: miR-203 expression in inducible keratinocytes**
P-values shown on chart are for miR-203

<table>
<thead>
<tr>
<th></th>
<th>miR-203</th>
<th>miR-205</th>
</tr>
</thead>
<tbody>
<tr>
<td>noninduced vs 6hr</td>
<td>0.0048*</td>
<td>Not Statistically Significant (NSS)</td>
</tr>
<tr>
<td>noninduced vs 24hr</td>
<td>0.0019**</td>
<td>NSS</td>
</tr>
<tr>
<td>noninduced vs 48hr</td>
<td>0.0005**</td>
<td>NSS</td>
</tr>
</tbody>
</table>

**Figure 8B: miR-203 induction with Calcium mediated differentiation**
no P-value data available

**Figure 9A: Impact of miR-203 expression on the cell cycle**
P values shown on chart are for G1/G0 and S

<table>
<thead>
<tr>
<th></th>
<th>G1/G0</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>0hr vs 6hr</td>
<td>0.0029*</td>
<td>0.0022*</td>
<td>NSS</td>
</tr>
<tr>
<td>0hr vs 12hr</td>
<td>&lt;0.0001***</td>
<td>&lt;0.0001***</td>
<td>NSS</td>
</tr>
<tr>
<td>0hr vs 24hr</td>
<td>&lt;0.0001***</td>
<td>&lt;0.0001***</td>
<td>NSS</td>
</tr>
<tr>
<td>0hr vs 48hr</td>
<td>&lt;0.0001***</td>
<td>&lt;0.0001***</td>
<td>NSS</td>
</tr>
</tbody>
</table>

**Figure 10A: Colony Formation**
P values shown on chart are for miR-203 induced vs noninduced

<table>
<thead>
<tr>
<th></th>
<th>day 1</th>
<th>day 3</th>
<th>day 4</th>
<th>day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>induced vs noninduced</td>
<td>NSS</td>
<td>&lt;0.0001***</td>
<td>&lt;0.0001***</td>
<td>&lt;0.0001***</td>
</tr>
</tbody>
</table>

**Figure 11B: Recovery**
P values shown are for total # colonies per well

<table>
<thead>
<tr>
<th></th>
<th>&gt;1.5mm</th>
<th>&lt;1.5mm</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0hr vs 24hr</td>
<td>0.0111*</td>
<td>NSS</td>
<td>NSS</td>
</tr>
<tr>
<td>0hr vs 48hr</td>
<td>&lt;0.0001***</td>
<td>NSS</td>
<td>0.0006**</td>
</tr>
<tr>
<td>0hr vs 72hr</td>
<td>&lt;0.0001***</td>
<td>&lt;0.0001***</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td>0hr vs continuous</td>
<td>&lt;0.0001***</td>
<td>&lt;0.0001***</td>
<td>&lt;0.0001***</td>
</tr>
</tbody>
</table>

**Figure 12A: miR-203 Induction**
P values shown are for 0hr vs 24 hr

<table>
<thead>
<tr>
<th></th>
<th>miR-203</th>
<th>miR-205</th>
</tr>
</thead>
<tbody>
<tr>
<td>0hr vs 24hr</td>
<td>&lt;0.0001***</td>
<td>0.0016*</td>
</tr>
</tbody>
</table>
**Figure 12A: miR-203 Induction**
P values shown are for 0hr vs 24 hr

<table>
<thead>
<tr>
<th></th>
<th>miR-203</th>
<th>miR-205</th>
</tr>
</thead>
<tbody>
<tr>
<td>0hr vs 24hr</td>
<td>&lt;0.0001***</td>
<td>0.0016*</td>
</tr>
</tbody>
</table>

**Figure 12B: miR-203 target gene expression**
P values shown are for 0hr vs 24 hr

<table>
<thead>
<tr>
<th>Gene</th>
<th>HPRT</th>
<th>Msi2</th>
<th>p63</th>
<th>Skp2</th>
<th>Vav3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0hr vs 24hr</td>
<td>NSS</td>
<td>0.0003**</td>
<td>0.0056*</td>
<td>0.0010**</td>
<td>0.0017*</td>
</tr>
</tbody>
</table>

**Figure 13A: Cell Cycle Rescue**
P values shown are for vector vs noninduced and vector vs Skp2

<table>
<thead>
<tr>
<th></th>
<th>vector vs noninduced</th>
<th>vector vs Msi2</th>
<th>vector vs p63</th>
<th>vector vs Skp2</th>
<th>vector vs Vav3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;0.0001***</td>
<td>0.0314</td>
<td>0.0132</td>
<td>0.0031*</td>
<td>NSS</td>
</tr>
</tbody>
</table>

**Figure 13B: Colony Formation Rescue**
No P values shown

<table>
<thead>
<tr>
<th></th>
<th>day 1</th>
<th>day2</th>
<th>day3</th>
<th>day4</th>
<th>day5</th>
</tr>
</thead>
<tbody>
<tr>
<td>vector vs noninduced</td>
<td>0.1752</td>
<td>0.0279</td>
<td>.0031*</td>
<td>.0063*</td>
<td>.0125*</td>
</tr>
<tr>
<td>vector vs Msi2</td>
<td>0.2212</td>
<td>0.4621</td>
<td>0.0831</td>
<td>0.078</td>
<td>0.1757</td>
</tr>
<tr>
<td>vector vs p63</td>
<td>0.0199</td>
<td>0.8672</td>
<td>0.5662</td>
<td>0.3527</td>
<td>0.2217</td>
</tr>
<tr>
<td>vector vs Skp2</td>
<td>0.8684</td>
<td>0.8768</td>
<td>0.8632</td>
<td>0.684</td>
<td>0.6156</td>
</tr>
<tr>
<td>vector vs Vav3</td>
<td>0.4257</td>
<td>0.7405</td>
<td>0.8946</td>
<td>0.8124</td>
<td>0.7772</td>
</tr>
</tbody>
</table>

**Figure 13D: Colony Formation Rescue**
P values shown are for total # colonies

<table>
<thead>
<tr>
<th></th>
<th>&gt;2mm</th>
<th>&lt;2mm</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>vector vs noninduced</td>
<td>&lt;0.0001***</td>
<td>&lt;0.0001***</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td>vector vs Msi2</td>
<td>N/A</td>
<td>0.0178</td>
<td>0.0178</td>
</tr>
<tr>
<td>vector vs p63</td>
<td>NSS</td>
<td>&lt;0.0001***</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td>vector vs Skp2</td>
<td>NSS</td>
<td>0.0037*</td>
<td>0.0052*</td>
</tr>
<tr>
<td>vector vs Vav3</td>
<td>NSS</td>
<td>0.0013**</td>
<td>0.0027*</td>
</tr>
</tbody>
</table>
**Figure 14A: Combinatorial Cell Cycle Rescue**

P values shown are for vec vs Skp2, vec vs ps, and vec vs mps

<table>
<thead>
<tr>
<th>Comparison</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>vector vs Msi2</td>
<td>NSS</td>
</tr>
<tr>
<td>vector vs p63</td>
<td>NSS</td>
</tr>
<tr>
<td>vector vs Skp2</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td>vector vs Vav3</td>
<td>NSS</td>
</tr>
<tr>
<td>vector vs Msi2+p63</td>
<td>NSS</td>
</tr>
<tr>
<td>vector vs Msi2+Skp2</td>
<td>NSS</td>
</tr>
<tr>
<td>vector vs p63+Skp2</td>
<td>0.0012**</td>
</tr>
<tr>
<td>vector vs Msi2+p63+Skp2</td>
<td>0.0017**</td>
</tr>
</tbody>
</table>

**Figure 14B: Combinatorial rescue of colony formation**

no P values shown

Preliminary data, no P values yet.
Supplementary Figure 1: BrdU Cell Cycle Analysis Gating.
A. Forward Scatter vs Side Scatter gate to select for cells and exclude debris. B. Forward Scatter vs Pulse Width gate to select for single cells and exclude doublets. C. FITC histogram gates to select GFP+ or GFP- cells. D. Violet 1 histogram to show Hoechst cell cycle profile. E. APC histogram to show BrdU+ and BrdU- populations. F. Violet 1 vs APC for final BrdU cell cycle analysis plot after gates have been applied.
Supplementary Figure 2: FACS Gating Profiles.
Red dots show cells that were sorted and used in assays. Black dots show cells that were selected against using the following gating scheme: A. Forward Scatter (X) vs Side Scatter (Y) to select for cells and against debris. B. Forward Scatter (X) and Side Scatter Width (Y) to select for single cells and against doublets. C. Histogram of Hoechst incorporation to select for live cells and against dead/dying cells. D. Histogram of GFP fluorescence to select for GFP+ cells. E. GFP (X) vs Hoechst (Y) to select for live, GFP+ cells. F. GFP vs GFP to select against autofluorescent cells.
Supplementary Figure 3: Recovery qPCR.

qPCR was used to determine the level of miR-203 expressed at the end of recovery. Cells were induced with doxycycline for 24 hours, then allowed to grow in the absence of doxycycline for 5 days. Continuously induced and noninduced samples were used as controls. After recovery, RNA was collected and analyzed using qPCR. Continuously induced cells had an 8-9 fold induction of miR-203, compared to the noninduced control. Recovery cells had a similar level of miR-203 expression at the end of the experiment as the noninduced control.