CIMRF SUPPRESSION OF ENDOGENOUS TISSUE DEVELOPMENT IN *CIONA INTESTINALIS* EMBRYOS

By:

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To

The Biology Department

Approved:

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Department Chair	Date

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Abstract

Trans-differentiation of non-muscle tissue to muscle tissue is a well-established property of vertebrate myogenic regulatory factors (MRFs). For instance the vertebrate MRF, MyoD, has been shown to convert nonmuscle cells to muscle cells. During this trans-differentiation, however, endogenous gene activity of the nonmuscle cells is down regulated. To test if this property is also found in invertebrate MRFs our lab has developed a system to analyze the ability of the MRF of *Ciona intestinalis*, *CiMRF*, to suppress endogenous gene activity during trans-differentiation. We did this by misexpressing *CiMRF* in the notochord and endoderm of *Ciona* embryos, two nonmuscle lineages, and by examining the expression of *Brachyury* and *Alkaline Phosphatase*, which are markers of notochord and gut differentiation respectively. Expression of *CiMRF* in the notochord of *Ciona* embryos reduced the level of *Brachyury* expression and resulted in embryos with abnormal tails. This latter result is consistent with studies demonstrating that tail development in *Ciona* requires *Brachyury* expression. In addition, expression of *CiMRF* in the endoderm led to a decrease in the level of alkaline phosphatase activity. These studies indicate that *CiMRF* functions similarly to vertebrate MRFs in its ability to suppress endogenous gene activity while directing muscle-specific gene activity in nonmuscle cell types.

Introduction

Myogenic regulatory factors (MRFs) are basic-helix loop helix (b-hlh) transcription factors that regulate muscle development. The expression of these transcription factors is highly regulated, occurring only in muscle or muscle precursor cells. This is important because when they are misexpressed by artificial means, MRFs can stimulate muscle development in non-muscle cell types (Baylies and Michelson, 2001; Pownall et al, 2002; Buckingham et al, 2003; Tajbakhsh, 2005; Tapscott, 2005; Meedel et al, 2007). Thus, it is important that MRF expression be highly regulated. Most studies of MRFs have been done with vertebrates, which have four MRF orthologs, as compared to invertebrates, which typically have only one MRF. My research was designed to shed further light on the functional relationship between vertebrate and invertebrate MRFs by using the MRF of the invertebrate chordate *Ciona intestinalis* as a model. *C. intestinalis* proves to be an ideal model organism as it shares traits from both groups of organisms. Similar to invertebrates, *C. intestinalis* has a single MRF termed *CiMRF*, however analogous to vertebrates this chordate requires its MRF to undergo myogenesis (Meedel et. al. 2007). Published work on vertebrate MRFs, such as mouse MyoD, has shown that these proteins are also capable of down regulating the endogenous program of gene expression when expressed in non-muscle cell types (Weintraub et al., 1989; Choi et al., 1990). Earlier work in Dr. Meedel's lab has demonstrated that *CiMRF* functions similarly to other MRFs in that it can direct muscle development in nonmuscle tissues (Meedel et al., 2007; Izzi et al, 2013). Here, I compare the properties of *CiMRF* to the vertebrate MRFs by investigating if *CiMRF* also down regulates endogenous developmental programs when it is expressed in nonmuscle tissues.

The vertebrate MRF, MyoD, has been shown to shut down endogenous gene activity (Rosenberg et al., 2006; Fulco et al., 2003). For instance, expression of MyoD in mouse

embryonic fibroblasts (MEFs) converted the fibroblasts to a skeletal muscle phenotype as expected. Interestingly, this conversion of fibroblast to skeletal muscle coincided with the inhibition of the follistatin-like 1 gene (Fstl1) and the Utrophin gene (Utrn), both of which have no well-defined biological role in muscle physiology but do in fibroblast physiology (Rosenberg et al., 2006). Earlier work in our lab has hinted at the possibility of *CiMRF* functioning in a similar manner to down regulate endogenous gene activity, but this notion was not fully tested. The work conducted by Izzi (2010) sheds light on this assertion. Here, the researcher expressed *CiMRF* in the notochord of *Ciona* embryos, which is of the mesodermal lineage. Following expression, muscle specific gene expression was noted in notochord. Another interesting note from Izzi's study was that abnormal tail formation was a result of this transformation of notochord to muscle. In *Ciona*, normal tail formation requires normal notochord formation, which in turn requires the expression of the brachyury gene [*Ci-Bra*] (Yasuo and Satoh, 1994). In the work presented in this paper we ask if the invertebrate MRF, *CiMRF*, functions similarly to the vertebrate MRF, MyoD in its ability to down regulate endogenous developmental programs.

We employed two main in vivo protocols in this study, termed the notochord assay and the endoderm assay. The first of the two tested the effects of expressing *CiMRF* in the notochord, a nonmuscle tissue, of *C. intestinalis.* For these studies, two previously constructed plasmids (pTLacZ and pTCiMRF) were utilized. Both of these plasmids contain cis-regulatory sequences that drive the expression of the encoded genes in the notochord (Corbo et al., 1997). In our research the LacZ gene served as a negative control as it has no known effect on gene expression. *CiMRF*, our experimental control, has been shown to elicit muscle expression in the tissues where it is expressed with the exception of those derived from the ectoderm (Meedel et

al., 2007; Izzi et al, 2013). Following electroporation, which we used to insert our plasmid of choice into *Ciona* embryos, embryos were allowed to develop for 12, 15, and 18 hours. Similar to the work of Izzi (2010), malformed tails were noted in these embryos. Previous work has shown that the disruption *brachyury* gene expression leads to abnormal tails in mice (Chesley 1935). Here, we investigated whether we can associate a loss of expression of *Ci-Bra*, which encodes a notochord-specific transcription factor that is essential for the formation of the notochord and thus indirectly, the tail, with malformed tails using a quantitative analysis.

In a different set of experiments, still involving the notochord assay, we tested if there is a decrease in *Ci-Bra* expression during notochord conversion to muscle on an individual cell basis. For this analysis electroporated embryos were cleavage arrested at the 64 cell stage. When allowed to develop up to tail formation following electroporation, *Ciona* embryos may appear distorted depending on the plasmid used. Furthermore, distinguishing the notochord and muscle lineages is not possible in embryos with highly abnormal tails. This problem was overcome by using embryos cleavage-arrested at the 64-cell stage where a clear distinction can be seen between the primary notochord, mesenchyme/secondary notochord, and muscle lineages (Figure 1). At the 64 cell

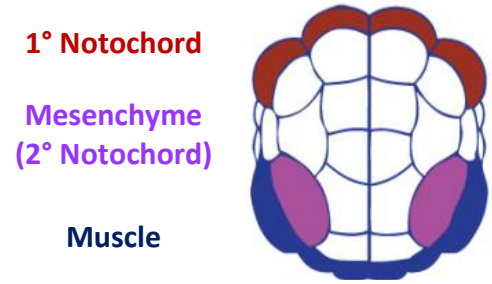


Figure 1 – Diagram illustrating the 64-cell stage of *Ciona* intestinalis embryo. Highlighted are three distinct cell lineages.

1. Primary notochord – indicated by four cells present at the apex of the embryo.
2. Mesenchyme/secondary notochord – indicated by two cells that are not uniquely fated.
3. Muscle – indicated by six cells present at the base of the embryo

stage there are 4 primary and 2 secondary notochord/mesenchyme lineage cells (note: these two cells are not uniquely fated at this stage and will give rise to both mesenchyme and notochord). If *CiMRF* expression disrupts *Ci-Bra* expression, and in turn notochord formation, then the number of notochord cells following in-situ hybridization should be fewer than 6. From this we were able to quantify the effect on *Ci-Bra* expression by expressing *CiMRF* in the notochord.

We next used the endoderm assay to explore the possibility that *CiMRF* affected other developmental programs such as larval gut formation. The plasmids used in these studies, pTTFLacZ and pTTFCiMRF, contain *cis* – regulatory sequences of *Ci-Titf1*, a gene that drives gut formation and is expressed only in the developing gut (Ristoratore et al. 1999). Following electroporation, embryos were subjected to an alkaline phosphatase histochemical test. In normally developing *Ciona* embryos, there is a strong localization of alkaline phosphatase activity in the gut. If *CiMRF* disrupted the development of this tissue, we hypothesize that it may also reduce the activity of this enzyme.

In this study an evaluation *CiMRF's* ability to drive ectopic muscle development and *CiMRF's* capacity to affect endogenous programs of gene expression is presented. In situ hybridization experiments showed that trans-differentiation of notochord cells to muscle cells by *CiMRF* significantly decreased *Ci-Bra* expression in the notochord. Additionally, *CiMRF* reduced the activity of alkaline phosphatase in the gut of *C. intestinalis* embryos. These results indicate that *CiMRF* functions similarly to vertebrate MRFs, such as MyoD, in its ability to down regulate endogenous developmental programs.

Materials & Methods

Plasmid Construction

Notochord Assay

A vector, termed pTReg and constructed by Dr. Meedel, was utilized to generate pTLacZ and pTCiMRF. pTReg contains the 3.3 kb cis regulatory region and the 5' untranslated region of the *Ciona intestinalis* brachyury gene (*Ci-Bra*) cloned into XhoI/PstI digested pSP72 (Promega) which has multiple cloning sites. *Ci-Bra* was chosen to create pTReg because it has been shown to express reporter genes in the notochord of *Ciona* embryos (Corbo et al., 1997). pTLacZ was generated by cloning a fragment of pSP72.127βgal (gift of R. Zeller) into pTReg. This fragment contains the E.coli LacZ gene and serves as a negative control in our experiments; it has no known effect on gene expression or notochord formation (Izzi 2010). pTCiMRF, which encodes full length *CiMRF*, has been shown to elicit muscle expression in the notochord of *Ciona* and serves as our experimental plasmid (Izzi, 2010; Izzi et al. 2013).

Endoderm Based Assay

The endoderm-based assay makes use of plasmids that contain the cis-regulatory sequence of the *CiTitf-1* gene, which encodes an endoderm-specific transcription factor (Ristoratore et al. 1999). Therefore if a sequence encoding *CiMRF*, for example, is placed downstream from the cis-regulatory sequence TTF, *CiMRF* will be expressed in the developing endoderm of *Ciona* embryos.

pTTFLacZ, which contains the *CiTitf-1* regulator sequences was obtained from Brad Davidson's lab. Analogous to pTLacZ, this plasmid served as our negative control. To construct pTTFCiMRF, a 2.1 kb fragment was excised from pTTFLacZ using SacI and re-ligated to generate pTTFΔSacI. NotI/Acc65I digested pTTFΔSacI and the NotI Acc65I insert of pBSCiMRF (containing the 5`and 3` untranslated regions (UTRs) and the full length *CiMRF*

mRNA sequence) were then ligated together to complete the construction of our test plasmid, pTTFCiMRF. The work described here was performed earlier in Dr. Meedel's Lab.

Electroporation Assay

Fertilization and Dechorionation

Three to five animals obtained from Point Judith, RI were used for each experiment. Eggs were surgically removed from the oviduct of these *Ciona* and eggs from individual animals were placed into a small dish of Millipore-Filtered Seawater (MFS) at a temperature of ~14°C or ~17°C. The eggs from the different animals were then pooled together in a filter basket and washed several times. These eggs were then transferred into a 50mL beaker with MFS at ~14°C or ~17°C and fertilized with a small amount of sperm obtained from the sperm duct of all the animals. After 5 minutes eggs were collected and washed in the filter basket, which has fine pores to hold the eggs but to allow the removal of sperm. The eggs were then collected in a corex tube and washed with 5mL Sodium Thioglycolate and Seawater (TSW; 0.5g Na Thoglycolate/50mL MFS) at pH 10 (85µL 5 N NaOH/10mL TSW). Following the wash, the fertilized eggs were gently collected using a hand-crank centrifuge and the TSW was aspirated off. 10µL of a 100 mg/ml stock of protease E was then added to 5mL of TSW and together added to the corex tube containing the fertilized eggs. This solution of TSW, protease E, and fertilized eggs was allowed to incubate until the eggs lost their chorions (microscopically verified). Dechorionated eggs were then washed 4 times with 5mL MFS by gentle centrifugating and decanting the seawater. Eggs were then suspended in MFS and transferred to a gel coated 60 mm petri dish (gel coating was made of a solution that contained 250mg of Knox gelatin in 25 mL of deionized water). Eggs were then divided into groups for electroporation with pTLacZ, pTCiMRF, pTTFLacZ, and pTTFCiMRF.

Electroporation and Development

25 micrograms of the selected plasmid DNA, 50µL 0.77M Mannitol, and 200µL concentrated eggs in seawater were placed in individual electroporation cuvettes. The solutions were then placed in an Electro Cell Manipulator and pulsed at ~50 volts for approximately 20msec. The solutions, containing the eggs, were then transferred to individual gel coated petri dishes containing MFS at ~14°C or ~17°C. Eggs at ~14°C were allowed to develop overnight for ~13hrs (mid-tailbud stage) and then culled for normal development. Eggs at ~17°C were allowed to develop for ~2.5 hrs (16-cell stage) and then culled for normal development. These embryos were used for 1) cleavage arrest studies and 2) tailbud developmental studies. Embryos that were used for cleavage arrest experiments were culled into gel-coated petri dishes containing 5mL MFS and raised until 4.25hrs post fertilization (64 cell stage). The cleavage of these embryos was then arrested with the addition of 2.5µL of 2µg/mL cytochalasin B. This solution containing the cleavage arrested embryos was then placed at ~17°C to allow development of the embryos until 12 hours post fertilization. For tailbud developmental studies, following culling, embryos were allowed to develop to early tailbuds formation (12 hrs), mid tailbuds formation (15 hrs), and late tailbuds formation (18 hrs).

Histochemistry

Fixation

Embryos that were used for alkaline phosphatase histochemistry were fixed in 4%PFA containing 0.1% Tween 80 in MFS solution for 30min on ice. Embryos were then washed three times with 500µL of phosphate-buffered saline solution and Tween80 (PBT_{80}; 1X PBS, 0.1% Tween 80).

Embryos subjected to in situ hybridization (embryos at 17°C) were fixed in 4% paraformaldehyde (PFA), 0.5M NaCl, 0.1M MOPS (pH 7.5) and 0.1% Tween 20 overnight at ~4°C. These embryos were then dehydrated through a series of graded ethanol/water solutions (25%, 50%, 75%, 100%EtOH) and stored at -20°C.

Alkaline phosphatase histochemistry

Alkaline phosphatase histochemistry was performed by staining embryos in a solution containing 50mM TrisHCl pH 9.5, 0.5M NaCl, 10mM $MgCl_2$, 0.1% Tween80, nitro blue tetrazolium chloride (NBT) @ 50mg/mL, and 5-bromo-4-chloro-3` indolyphosphate p-toluide salt (BCIP) @ 100mg/mL for 1 hour. Embryos were later stored in 4% PFA PBT_{80} at 4°C. The oxidation of the indolyl substrate by alkaline phosphatase ultimately results in the release of hydrogen, which reduces the tetrazolium salt to diformazan (blue colored) that is easily visualized.

In situ hybridization

In situ hybridization (ISH) encompassed a three day protocol. Day one consisted of rehydrating the dehydrated embryos through a series of graded ethanol/phosphate-buffered saline and $Tween_{20}$ (PBT_{20}) washes (100%, 75%, 50%, 25% EtOH/PBT_{20}). Following rehydration embryos were washed three times in PBT_{20} then incubated in 100µL 2µg/mL proteinase K/PBT_{20} for 10min at room temperature (RT). Immediately after, the embryos were rinsed three times with PBT_{20} and post fixed in a solution containing 4% PFA in 1X PBS for 1 hour at RT. Embryos were then washed twice in PBT_{20} and incubated in 100µL hybridization buffer (50% formamide, 6X SSC, 2.5X Denhardt's, 1% tRNA @ 20mg/mL, 0.1% $Tween_{20}$) for 10min at RT. The embryos were then placed in fresh hybridization buffer and incubated at 55°C for 1 hour. Following the removal of the hybridization buffer, the embryos were incubated overnight (18hrs)

in 100µL of hybridization buffer containing Digoxigenin U (Dig U)-labeled antisense RNA probe (500ng/mL) at 55°C. Day two began with the removal of the hybridizztion solution followed by a series of four washes at 55°C (Wash 1 - 50% formamide, 5X SSC, 1% SDS; Wash 2 - 50% formamide, 2X SSC, 1%SDS, dH2O; Wash 3 - 2X SSC, 0.1% Tween 20; Wash 4 - 0.2X SSC,0.1% Tween 20, dH2O). Note: each wash was performed twice using 500µL of wash solution. Embryos were then placed in PBT_{20} for 5 minutes at RT then in Dig blocking solution (0.1M Tris pH 7.5, 0.15M NaCl, 0.5% Boehringer Blocking Reagent) for 1hr at RT. To conclude day two, embryos were placed in a new aliquot of Dig blocking solution containing anti-DigU antibody (1/2000 dilution) overnight at 4°C. The final day of in situ hybridization began with four PBT_{20} washes each for 1 hour at RT followed by two washes in TMNT buffer (100mM NaCl, 50mM MgCl2, 100mM Tris-Cl pH 8.0, 0.1% Tween 20). Next the embryos were placed in TMNT buffer containing 2mM Levamisole for 5 minutes at RT. To conclude the ISH protocol, embryos were incubated in the dark in the color development solution (TMNT-Levamisole, NBT @ 50mg/mL, BCIP @ 100mg/mL) at RT for several hours to overnight. Stained embryos were then stored in 4% PFA PBT_{20} at 4°C.

Embryo Assessment

Following ISH embryos were examined using a Leica dissecting microscope following three washes with PBT. pTLacZ electroporated embryos were compared with pTCiMRF electroporated embryos in order to assess potential differences in the ability of these constructs to affect normal tail development and *Ci-Bra* expression. Results from pTTFLacZ electroporated embryos and pTTFCiMRF embryos were compared to determine differences in alkaline phosphatase activity in the two samples.

Cleavage arrested embryos

pTLacZ and pTCiMRF electroporated embryos were assessed based on the number of embryos that expressed a particular marker (Table 1).

Table 1
Muscle marker genes referenced in this study.

Abbreviation	Gene
CiBra	*C. intestinalis* Brachyury
CiMRF	*C. intestinalis* MRF
TnI	Troponin I

12hr, 15hr, and 18hr embryos

Following ISH embryos were evaluated to determine if there was a correlation with notochord cells expressing *Ci-Bra* and normal tails or notochord cells not expressing *Ci-Bra* and abnormal tails.

Alkaline phosphatase embryos

Embryos were examined every 15 minutes during the 1 hour staining processes based on the intensity of staining present in the gut region.

Statistical Analysis

A Fisher's Test of a 2 by 2 contingency table was used to determine statistically significant differences between samples. The test allowed a quantitative comparison between the numbers of embryos expressing a particular marker in response to the experimental or negative control plasmids. In these studies a p-value of 0.05 or less was considered significant.

Photography

Photographs were taken in two different ways. ISH photographs were taken using an Olympus System BHS Model microscope with a Pixelink 6.6 megapixel camera. Images were then digitized with the Pixelink Image Capture software. Photographs of alkaline phosphatase-stained embryos were taken using a Leica dissecting microscope, equipped with a Leica DFC290 HD digital camera with 3 Megapixel standard resolution. These photographs were then digitized using Leica Application Suite (LAS) software. ImageJ was used on all photographs for editing purposes.

Results

For each experiment between 100 and 200 normally developing embryos were culled per plasmid following electroporations. This allowed for a sufficient number of embryos when investigating all four plasmids.

Tail malformation is correlated with a decrease in the expression of the brachyury Gene

Tail formation requires the normal development of the notochord, a mesodermal structure that requires the expression of the *Ci-Bra* gene (Yasuo and Satoh, 1994). Therefore, a decrease in the expression of *CiBra* should correlate with abnormal tail formation. To investigate this possibility, embryos electroporated with pTLacZ and pTCiMRF were grown for 12 hours (early tail bud), 15 hours (mid tail bud), and 18 hours (late tail bud) and then examined for *Ci-Bra* expression. All pTLacZ electroporated embryos assayed for *CiBra*, regardless of developmental stage, expressed the gene (Figure 2A – 2C). Compared to embryos electroporated with pTLacZ, *CiBra* expression, was significantly reduced and tails were typically malformed in embryos electroporated with pTCiMRF (Figure 2D – 2F; Table 2).

Table 2

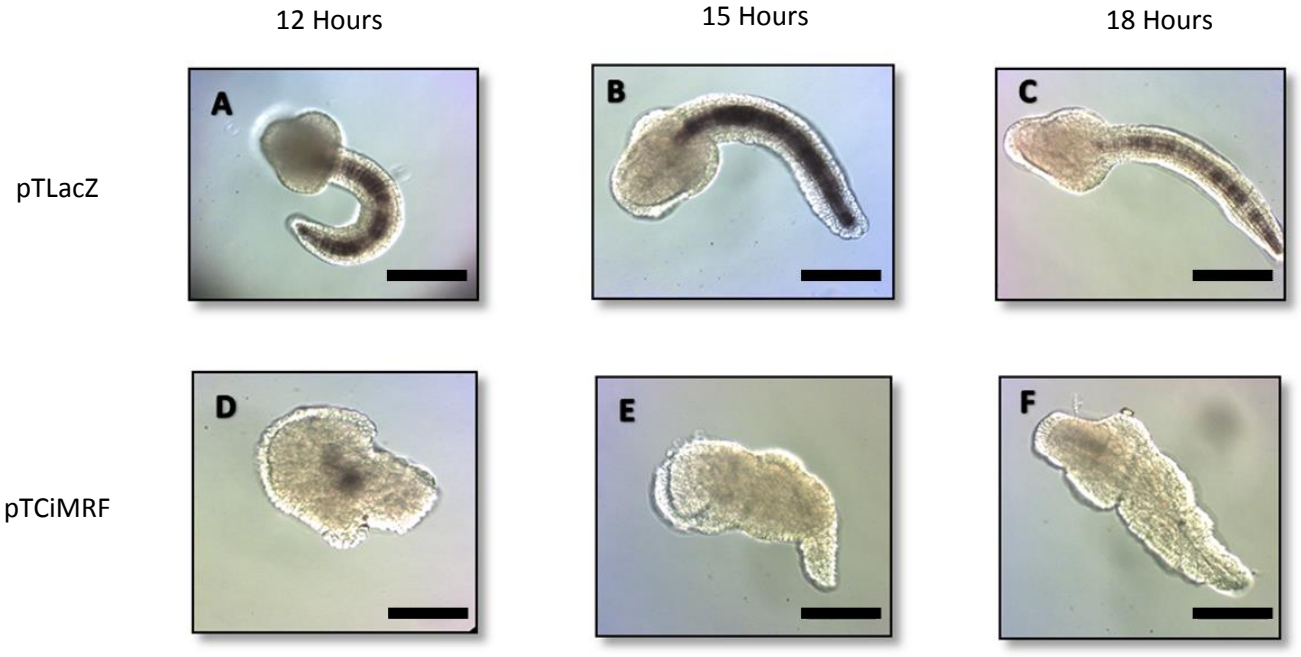
Percentage of embryos expressing CiBra at the indicated time post-fertilization.

		pTLacZ	vs.	**pTCiMRF**
Marker	Time Post-fertilization		p-value	
CiBra	12 h	100% [60/60] (3)	**0.0001**	21.95% [9/41] (3)
	15 h	100% [49/49] (3)	**0.0001**	22.86% [8/35] (3)
	18 h	100% [34/34] (3)	**0.0001**	20.83% [5/24] (3)

Percentages indicate the proportion of embryos that expressed *Ci-Bra*. Brackets specify the number of embryos expressing/total number of embryos. Parentheses designate the number of experiments performed. P-values indicate the statistical significance of the differences between pTLacZ and pTCiMRF electroporated embryos probed with *CiBra*.

Figure 2

Examples of staining in 12, 15, and 18 hour embryos (plasmid/marker). A – C) pTLacZ/CiBra; 12, 15, 18 hour respectively. D – F) pTCiMRF/CiBra; 12, 15, 18 hour respectively. Scale bar 150µm.



pTCiMRF converts notochord to muscle by down regulating brachyury in cleavage arrested embryos

As expected, we also found that *CiMRF* is capable of converting notochord cells to muscle cells (Figure 3A – 3C). In two separate sets of experiments embryos electroporated with pTCiMRF expressed muscle markers TnI and *CiMRF* at levels that were significantly different than embryos electroporated with pTLacZ. In fact, essentially none of the pTLacZ-electroporated embryos expressed these muscle markers in the notochord as expected (Table 3). When pTLacZ-electroporated embryos were probed with *Ci-Bra* all of the embryos expressed the *Ci-Bra* gene. This gene was down regulated when the experimental plasmid, pTCiMRF, was used (Figure 3D – 3E). This difference also deemed significant (Table 4). Normally there are 4 primary and 2 secondary notochord lineage cells in cleavage arrested 64 cell *C. intestinalis* embryos. The expression of CiMRF in the notochord lineage reduced the mean number of Ci-Bra expressing cells to less than half the number observed in embryos expressing LacZ in the notochord (Table 5).

The results seen in table 4 and table 2 are very different. In table 2 the embryos were not cleavage arrested while in table 4 they were. This may be one possible explanation for the difference. Another possibility for this difference may be the conditions of electroporations in table 2 and table 4. Results in table 4 were obtained using an older lab protocol in which the plasmid concentration in electroporations was lower than what we currently use, and which was used in the experiments detailed in table 2. Using the higher concentration of plasmids is expected to result in embryos incorporating more plasmid DNA as well as a higher percentage of embryos being transformed. This changed protocol may also be responsible for some or all of the differences between the results seen in the two tables.

Table 3

Percentage of cleavage arrested embryos expressing the designated muscle marker following ISH of pTLacZ and pTCiMRF electroporated embryos.

	pTLacZ	vs.	**pTCiMRF**
Marker		p-value	
TnI	4.22% [6/142] (3)	**0.0001**	86.96% [100/115] (3)
CiMRF	3.48% [4/115] (3)	**0.0001**	81.20% [95/117] (3)

Percentages indicate the proportion of embryos that expressed the particular muscle marker. Brackets specify the number of embryos expressing/total number of embryos. Parentheses designate the number of experiments performed. P-values indicate the statistical significance of the differences between pTLacZ and pTCiMRF electroporated embryos.

Table 4

Percentage of cleavage arrested embryos expressing CiBra following ISH of pTLacZ and pTCiMRF electroporated embryos.

	pTLacZ	vs.	**pTCiMRF**
Marker		p-value	
CiBra	100% [182/182] (3)	**0.0001**	68.28% [99/145] (3)

Percentages indicate the proportion of embryos that expressed *Ci-Bra*. Brackets specify the number of embryos expressing/total number of embryos. Parentheses designate the number of experiments performed. P-values indicate the statistical significance of the differences between pTLacZ and pTCiMRF electroporated embryos probed with *CiBra*.

Table 5

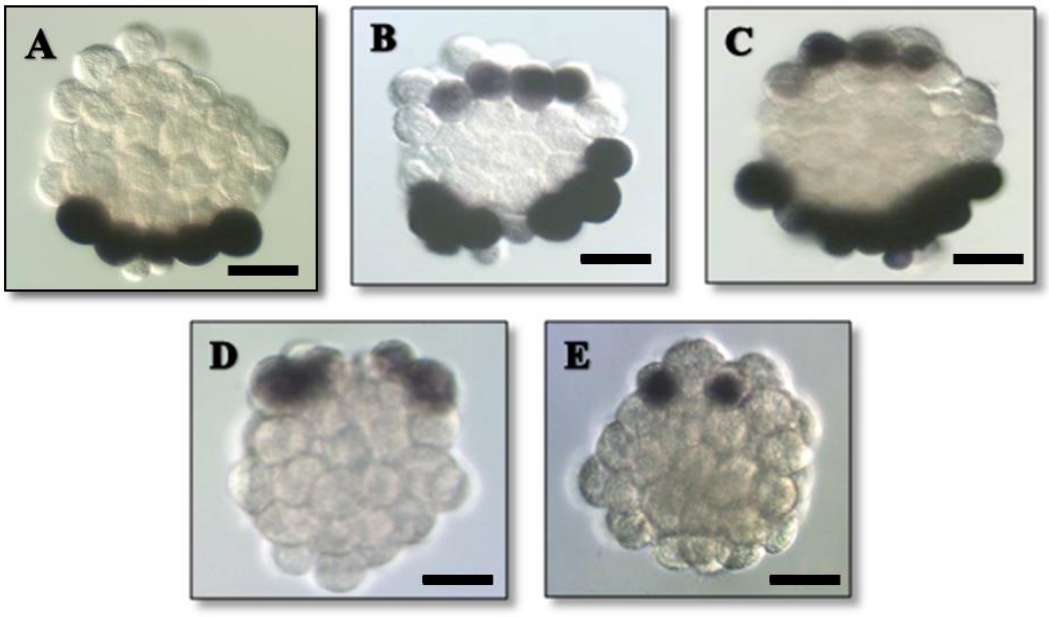
Notochord cells expressing CiBra following ISH of cleavage arrested embryos electroporated with pTLacZ and pTCiMRF.

Number of notochord cells expressing *CiBra*	Number of **pTLacZ** electroporated embryos	Number of **pTCiMRF** electroporated embryos
0	0	46
1	4	25
2	6	45
3	21	14
4	151	15
Total	182	145
Mean number of notochord cells expressing *CiBra*	**3.75 ± 0.62**	**1.50 ± 1.31**

The number of primary notochord cells capable of expressing *CiBra* is between 0 and 4. The middle column indicates the number of pTLacZ electroporated embryos that expressed a particular number of *CiBra* positive cells (e.g. 151 embryos electroporated with pTLacZ expressed *CiBra* in four notochord cells). The right hand column indicates the number of pTCiMRF electroporated embryos that expressed a particular number of *CiBra* positive cells. The last row indicates the mean number of notochord cells per plasmid plus/minus the standard deviation.

Figure 3

Examples of staining in cleavage arrested embryos (plasmid/marker). A)pTLacZ/TnI. B) pTCiMRF/TnI. C) pTCiMRF/*CiMRF*. D) pTLacZ/CiBra. E) pTCiMRF/CiBra. Scale bar 50μm.



Expressing CIMRF in the Gut reduces Alkaline Phosphatase activity

Alkaline phosphatase, an enzyme that is abundantly expressed in the gut of *Ciona*, is responsible for removing phosphate groups from many types of molecules. In our assay, the substrate for this enzyme was BCIP and its dephosphorylation leads to the production of a blue/violet color in the gut region of the *Ciona* embryos. As a result the degree to which this blue/violet color is produced is indicative of the activity of this enzyme, which we interpret to be correlated with its concentration. In short, little to no staining indicates low alkaline phosphatase activity (and low levels of enzyme) while darker staining indicates higher enzyme activity (and higher levels of enzyme). The majority of pTTFLacZ-electroporated embryos showed dark staining while the majority of those electroporated with pTTFCiMRF showed little or no staining (Table 6; Figure 4).

Table 6

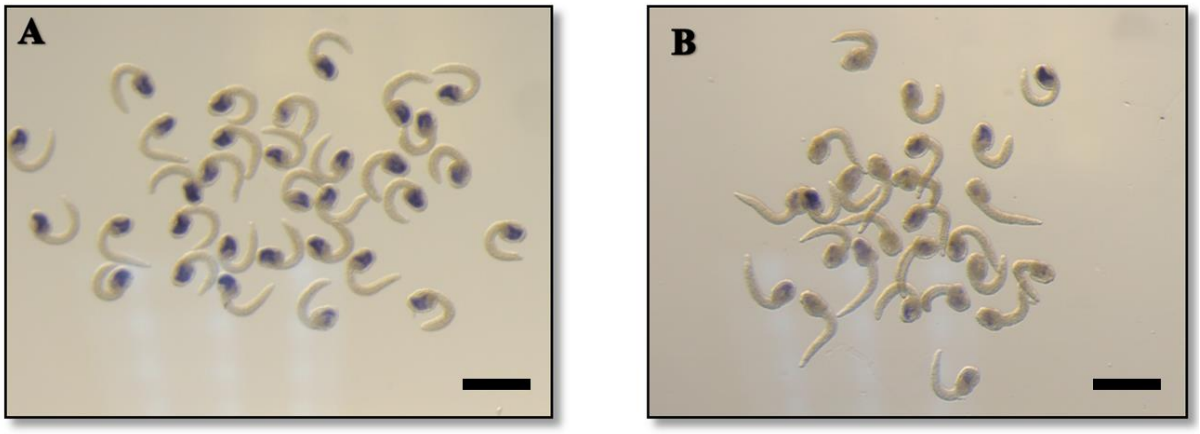
Embryos showing alkaline phosphatase activity following histochemistry.

	pTTFLacZ	vs.	**pTTFCiMRF**
		p-value	
Dark Stain	252	**0.0001**	76
Light Stain	1		167

Dark stain represents a high level of alkaline phosphatase activity. Light stain represents a low level of alkaline phosphatase activity. Four experiments were conducted for each plasmid. P-values indicate the statistical significance of the differences between pTTFLacZ and pTTFCiMRF electroporated embryos on level of alkaline phosphatase activity.

Figure 4

Alkaline phosphatase activity in electroporated *C. intestinalis* embryos. A)pTTFLacZ electroporated. B) pTTFCiMRF electroporated. Scale bar 500µm.



Discussion

During trans-differentiation mature somatic cells transform to other mature somatic cells. For this to occur, the endogenous gene activity of a cell must be down regulated. For instance, during the conversion of nonmuscle cells to muscle cells by the vertebrate MRF, MyoD, endogenous gene activity of the nonmuscle cells is down regulated (Rosenberg et al., 2006; Fulco et al., 2003). In this study I investigated whether the invertebrate MRF of *Ciona intestinalis*, *CiMRF*, could act in a similar manner to suppress endogenous gene activity while converting nonmuscle cells to muscle cells.

Previous work in the lab has demonstrated the ability of *CiMRF* to direct muscle development in nonmuscle cell types. In particular, expression of *CiMRF* in the notochord or in the endoderm of *Ciona* embryos results in the conversion of the notochord or endoderm to a muscle-like phenotype (Meedel et al., 2007; Izzi et al, 2013; Pickett 2013). In the case of the vertebrate MRFs, expression of MyoD led to the conversion of these mouse embryonic

fibroblasts to skeletal muscle. This conversion corresponded with the inhibition of two genes, Fstl1 and Utrn, which are expressed in fibroblast cells (Rosenberg et al., 2006).

Earlier work by Izzi (2010) showed that expression of *CiMRF* in the notochord of *Ciona* embryos corresponded with abnormal tail formation during trans-differentiation. Normal tail formation in ascidians is dependent on normal notochord formation, which, in turn, is dependent on the *Ci-Bra* activity (Yasuo and Satoh, 1994). Therefore, it is possible that during trans-differentiation, *CiMRF* down regulates endogenous developmental programs by inhibiting *Ci-Bra* expression.

We confirmed that abnormal tail formation is correlated with the expression of *CiMRF* in the notochord of *Ciona* embryos. At various tail developmental stages, *Ci-Bra* expression was examined in both control embryos (pTLacZ electroporated) and experimental embryos (pTCiMRF electroporated). In control embryos *Ci-Bra* expression was noted at all developmental stages examined. *Ci-Bra* expression, however, was significantly reduced at all developmental stages in experimental embryos when compared to control embryos. Furthermore the lack of *Ci-Bra* expression correlated with malformed tails in experimental embryos. These results demonstrate that abnormal tail formation is correlated with a decrease in the expression of the *Ci-Bra* gene.

The influence of *CiMRF* on the notochord was also assessed in cleavage-arrested embryos. *CiMRF* converts notochord to muscle. Here, we used TnI and *CiMRF* as markers for muscle cell differentiation. Virtually no embryos electroporated with pTLacZ misexpressed TnI or *CiMRF* in the notochord indicating that notochord was not converted to muscle as expected. The low percentages of embryos misexpressing TnI or *CiMRF* in control embryos most likely represents staining of secondary muscle lineage cells, aberrant rearrangement of muscle lineage

cells during cleavage arrest, or some combination of the two. The observation that we never saw misexpression of muscle specific genes in the endoderm of embryos electroporated with pTTFLacZ supports our contention that low level misexpression of muscle-specific genes in what we took to be the notochord lineage of cleavage-arrested embryos electroporated with pTLacZ is an artifact of the experimental protocol. In comparison with pTLacZ electroporated embryos, there was a significant increase in the expression of TnI or *CiMRF* in the notochord of pTCiMRF electroporated embryos indicating that the notochord was transformed to a muscle phenotype as previously observed. In addition, in these embryos the expression of *Ci-Bra* was significantly reduced compared to control embryos, which all contained *Ci-Bra* positive notochord lineage cells. Consistent with this pTLacZ electroporated embryos showed a greater mean number of *Ci-Bra* positive notochord cells when compared to pTCiMRF electroporated embryos. These results indicate that during the conversion of notochord to muscle, *CiMRF* down regulates the expression of *Ci-Bra*.

Previous work in the lab has shown that CiMRF can convert the larval gut (endoderm) of *Ciona* to muscle (Pickett 2013). Here, we questioned if this conversion was also accompanied by down regulation of endodermal developmental programs. The endoderm of *Ciona* embryos has a high concentration of alkaline phosphatase that makes this enzyme an excellent marker of gut differentiation. Therefore if *CiMRF* disrupts endogenous developmental programs, then during trans-differentiation the activity of this enzyme should be reduced in experimental embryos electroporated with pTTFCiMRF when compared to control embryos electroporated with pTTFLacZ. The results obtained in this study supported this prediction. The level of alkaline phosphatase activity in experimental embryos was significantly reduced in comparison

to its activity in control embryos. These results indicate that during the conversion of endoderm to muscle, *CiMRF* down regulates the activity of alkaline phosphatase.

Taken together, my results show that like vertebrate MRFs, *CiMRF* also down-regulates endogenous developmental programs during trans-differentiation. For example, during trans-differentiation of fibroblasts to muscle the expression of Fstl1 and Utrn is reduced and this is accompanied by induction of miR-206, a muscle specific microRNA, by MyoD. miR-206, in turn, targeted specific sequences in Fstl1 and Utrn mRNAs leading to the post-transcriptional suppression of the activity of these genes, which is associated with fibroblast formation. These results indicate that not only does MyoD activate muscle specific genes during trans-differentiation but that it induces microRNAs that down regulate endogenous gene activity (Rosenberg et al., 2006).

In this study, we demonstrated the effects of *CiMRF* on endogenous gene programming during trans-differentiation of two non-muscle cell types to cells that exhibited a muscle phenotype. However, the pathway through which *CiMRF* to suppress this process remains unclear. Perhaps *CiMRF* down regulates endogenous notochord and gut development by activation of microRNA expression in similar ways as does MyoD. Testing this possibility might be a fruitful area of future study in the lab.

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