Construction and Development of a Cardiac Tissue-Specific and Hypoxia-Inducible Expression Vector

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Abstract
Purpose: Cardiovascular gene therapy is a sophisticated approach, thanks to the safety of vectors, stable transgene expression, delivery method, and different layers of the heart. To date, numerous expression vectors have been introduced in biotechnology and biopharmacy industries in relation to genetic manipulation. Despite the rapid growth of these modalities, they must be intelligently designed, addressing the cardiac-specific transgene expression and less side effects. Herein, we conducted a pilot project aiming to design a cardiac-specific hypoxia-inducible expression cassette.

Methods: We explored a new approach to design an expression cassette containing cardiac specific enhancer, hypoxia response elements (HRE), cardiac specific promoter, internal ribosome entry site (IRES), and beta globin poly A sequence to elicit specific and inducible expression of the gene of interest. Enhanced green fluorescent protein (eGFP) was subcloned by BglII and NotI into the cassette. The specificity and inducible expression of the cassette was determined in both mouse myoblast C2C12 and mammary glandular tumor 4T1 as ‘twin’ cells. eGFP expression was evaluated by immunofluorescence microscope and flow cytometry at 520 nm emission peak.

Results: Our data revealed that the designed expression cassette provided tissue specific and hypoxia inducible (O2<1%*) transgene expression.

Conclusion: It is suggested that cardiac-specific enhancer combined with cardiac-specific promoter are efficient for myoblast specific gene expression. As well, this is for the first time that HRE are derived from three well known hypoxia-regulated promoters. Therefore, there is no longer need to overlap PCR process for one repeated sequence just in one promoter.

Introduction
Since identification and description of hypoxia-inducible factor 1 (HIF-1) by Wang et al.,1 more than 200,000 articles have been published in scientific literatures. HIF-1 is an inducible transcription factor, which binds to hypoxia response elements (HREs) or enhancer elements during hypoxia. HREs are located in the upstream of promoter region. HIF-1 regulates several genes, such as vascular endothelial growth factor (VEGF) and erythropoietin (EPO).2 hypoxia inducible factor-1 alpha (HIF-1) is comprised of a heterodimeric basic helix-loop-helix (bHLH) transcriptional complex and is divided into distinct subsets, including HIF-1α and aryl hydrocarbon receptor nuclear translocator (ARNT), encoding a protein that is referred to beta (β) subunit. HIF-1 belongs to a conserved subfamily of PER-ARNT-SIM (PAS), which functions as oxygen sensors. The PAS domain is a subfamily of bHLH, which is a transcription factor. The HIF family consists of HIF-1α, -1β, -2α, -2β, -3α, -3ß.3,4 In normoxia, HIF-1α is degraded by ubiquitin-mediated proteolysis activity, while being transcribed into mature DNA under hypoxic conditions, leading to an increase in oxygen delivery to the tissues. Therefore, HIF-1α has an essential role both in physiologic and pathologic conditions, including, but not limited to, myocardial ischemia, coronary artery disease, organ rejection and some cancers.5,6

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Among all the above-mentioned circumstances, HIF-1α plays a great role in cardiovascular diseases. Lack of oxygen contributes to augmented hypoxia in coronary and cardiac tissue, subsequently leading to a cardiac stroke. Despite the emergence of novel surgical and medical approaches, cardiac infarction is set to become the leading cause of death up to the year 2020.7 Several reports have indicated that cardiac function, if not completely, then partially, can be ameliorated following gene or cell therapy in experimental models of infarction.8,9 However, ectopic gene expression has its disadvantages, such as hemangiomia or uncontrolled vascular formation. It is, therefore, essential to regulate gene expression via the modulation of upstream elements of promoters like HREs. It should also be noted that hypoxia may subsequently exist in other tissues as a result of physiologic statement. Hence, tissue specific gene expression is essential to develop suitable vectors for gene therapy. This approach is also considered to be useful for sophisticated studies in the field of biomedical research such as cellular imaging and tracking.10 genome editing,11 stem cell engineering,12 and the study of signaling pathways.13,14 In this study, we investigated the cardiac specific promoter and cardiac specific enhancer combined with HREs consensus sequence.

**Materials and Methods**

**Expression cassette designing and cloning**

In order to construct the expression cassette, all the elements were synthesized by Generay Biotechnology Company (Shanghai, China) and sub-cloned into pGH cloning vector by Ndel and NheI restriction enzymes (Fermentas, Germany) (Figure 1). eGFP was sub-cloned into the cassette by BglII and NotI (Figure 2). Briefly, 1 µg plasmid DNA was digested by BglII and NotI (Fermentas, Germany) and the relevant buffer was added up to the final volume of 20 µl in 37 °C for 1 h. Enzymatic reaction was inactivated by chloroform and the cloning procedure was confirmed by HindIII digestion.

![Figure 1. Schematic view of expression cassette. Expression cassette contains CASQ2 enhancer, HRE, MLC2v promoter, IRES, and β-globin poly A sequence.](image1)

![Figure 2. Schematic view of eGFP cloning into the cassette. eGFP was cloned into the cassette by NotI and BglII restriction enzymes.](image2)

**Bacterial strains and plasmid preparation**

Two strains of *Escherichia Coli* (E. coli) were used including DH5α and DH10β (Top10, Invitrogen, Thermo Fisher Scientific, USA). The plasmid was transformed into cells using the calcium chloride (CaCl2) method.16 E. coli bearing the desired plasmid was cultured in lysogeny broth (LB) media for 16 hrs at 37 °C in a shaker incubator. Cells with a density of 3-4×10⁸ cells/ml with OD₆₀₀=3 were harvested and DNA was extracted by plasmid DNA extraction kit (Qiagen, Midiprep Plasmid DNA Extraction Kit).

**Cell Culture**

The mouse myoblast cell line C2C12 (CRL-1772) and the mouse mammary gland cell line 4T1 (CRL-2539) was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). C2C12 cells were cultured in high glucose Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% fetal bovine serum (FBS, Invitrogen, USA) and 0.584 g/L L-glutamine (Sigma-Aldrich, USA) in 37°C incubator and humidified 5% CO₂ and 95% air.17 4T1 cells, a 6-thioguanine resistant cell line,18 were cultured in RPMI-1640 (Sigma-
Aldrich, USA) and 10% FBS (Invitrogen Gibco) in 37°C incubator and humidified atmosphere with 5% CO₂ and 95% air. Cell lines were passaged after 80% confluence.

The induction of hypoxic condition
To create hypoxia, C2C12 and 4T1 cell lines were incubated for 90 min in a hypoxic condition, containing 94% N₂, 4% CO₂ and 1% O₂, and hanks buffer. As a normoxia control “twin” cells were kept in a normoxic incubator.

Plasmid Transient Transfection
For achieving transient transfection, Gene Pulser XcellTM electroporation system (Bio-Rad, USA) was used. Following 60% confluence, media was removed and cells were harvested using 0.25% Trypsin-EDTA solution. Approximately 4-5 ×10⁶ cells were harvested by adding 3 ml complete media and then centrifuged for 5 min at 1000g and 4°C.¹⁹ Thereafter, cells were re-suspended in 400µl opti-MEM (buffer O).²⁰ Then, 10µg of supercoiled DNA was overlaid to the cell suspension and mixed in wells. The cuvette was placed on ice for 5 min. Then, cells were transfected with the optimized exponential protocol (one shock for 18 seconds, at a voltage 120).

Fluorescence microscopy
Slides were visualized with a Zeiss Axioplan using 485 band pass filters set to view eGFP. All images were analyzed with AxioCam digital camera and Zeiss proprietary software (Axiovision Ver. 3.0.6.0). Images were manipulated in Adobe Photoshop 5.5.

Flow cytometry
eGFP expression was detected 48 hrs after transfection. Myocyte cells were harvested by trypsin/EDTA. Cells were centrifuged at 1000g for 10 min at 4°C. The cells were then washed three times with 500 µl of PBS. Fluorescence-activated cell sorting (FACS) caliber-micro flow cyrometer (Becton Dickinson, NJ, USA) was used to analyze eGFP expression. GFP was excited by an argon laser and fluorescence’s at 485/520 nm band pass filter in the FL1 channel. All raw data were analyzed using FlowJo software version 7.6.1.

Western Blotting
Cells in the both hypoxia and normoxia groups were collected from the wells and their protein contents were detected using an extraction Kit (Santa Cruz, USA) following the manufacturer’s protocol. Total protein concentration was measured using a Nanodrop (Thermo-Scientific, USA). Samples were prepared for western blotting by adding loading buffer to each sample. Proteins were electrophoresed on 12% SDS-polyacrylamide gel and transferred to PVDF membranes. The membranes were blocked by incubating with 0.3 g bovine serum albumin in 10 ml of washing buffer at 4°C overnight. Membranes were then washed three times with PBS for 10 min. Then, the membranes were incubated with anti-HIF-1α antibody (dilution: 1:500; Santa-Cruz) for 4 hrs at 4°C. The membranes were then washed three times for 10 min each and incubated with the secondary antibody for 2 hrs. Roche ECL kit and semi-dry X-ray were used for imaging of immunoreactive protein bands.

Results
eGFP Cloning confirmation with HindIII digestion
To confirm eGFP cloning, the expression cassette was digested by HindIII and positive colony was determined by three sharp plasmid DNA band, including 3000, 2000 and 1500 bps on gel electrophoresis. Meanwhile, empty expression cassette was digested into two bands on 2500 and 1500 bps (Figure 3A and B).

Exploration of the CASQ2 enhancer and cardiac tissue specific promoter
Calsequestrin 2 (CASQ2) as a cardiac specific enhancer plus myosin light chain-2 (MLC2v) cardiac specific promoter provided tissue specificity. Thanks to highly cardiac specific sequence expression, eGFP was detected in myoblast C2C12 cell line, but not in 4T1 ‘twin’ cell.

Exploring of HRE
In this study, 35 putative sites, including 9 ARNT, HIF-1, and 26 sp1 from EPO, VEGF-A, and phosphoglycerate kinase 1 (PGK-1) promoters were driven (Figure 4A and B, Table 1). HRE sequence was analyzed for insulator boundary elements to prevent enhancer and promoter interaction (Table 2). The elements provided hypoxia inducible expression. eGFP was detected under hypoxia and was not expressed under normoxia condition.
Hypoxia induction in C2C12 and 4T1 cell lines
HIF-1α protein at transcriptional and translational levels was stabilized under hypoxic condition. Then, we detected HIF-1α protein in hypoxic condition. Notably, we could not detect any HIF-1α protein in normoxia (Figure 5C).

Table 1. 35 Putative sites that is existing on Hypoxia Responses Elements (HRE) including 9 ARNT and 26 sp1.

| Model ID | Model name | Score | Relative score | Start | End | Strand | predicted site sequence |
|----------|------------|-------|----------------|-------|-----|--------|-------------------------|
| MA0079.1 | SP1        | 6.736 | 0.800936630165687 | 27    | 36  | 1      | CCCCCGGGAC              |
| MA0079.3 | SP1        | 1.668 | 0.802126030842755 | 38    | 48  | 1      | CTTCCGTGCTC             |
| MA0079.3 | SP1        | 14.558 | 0.964296987823859 | 44    | 54  | 1      | GCTCCGCCCCCT            |
| MA0079.2 | SP1        | 9.501 | 0.870571327605649 | 45    | 54  | 1      | CTCGGCCTCC              |
| MA0079.1 | SP1        | 6.122 | 0.800408014237765 | 46    | 55  | -1     | TAGGGGCGGA               |
| MA0259.1 | ARNT::HIF1A| 8.788 | 0.928072894030867 | 81    | 88  | 1      | CGCCGTGC                |
| MA0259.1 | ARNT::HIF1A| 9.817 | 0.958770979367351 | 90    | 97  | 1      | GACGTTG                 |
| MA0259.1 | ARNT::HIF1A| 10.396 | 0.976043464702457 | 110   | 117 | -1     | AGACGGTC                |
| MA0259.1 | ARNT::HIF1A| 9.025 | 0.935143183364926 | 145   | 152 | 1      | ATACGGTG                |
| MA0259.1 | ARNT::HIF1A| 10.321 | 0.973806031368894 | 174   | 181 | 1      | CTACGGTC                |
| MA0079.1 | SP1        | 7.476 | 0.847637728498468 | 188   | 197 | -1     | CAGGCTGGT                |
| MA0079.2 | SP1        | 6.736 | 0.800936630165687 | 212   | 221 | 1      | CCCCCGGGAC               |
| MA0079.3 | SP1        | 1.668 | 0.802126030842755 | 223   | 233 | 1      | CTTCCGTGCTC             |
| MA0079.3 | SP1        | 14.558 | 0.964296987823859 | 229   | 239 | 1      | GCTCCGCCCCCT            |
| MA0079.2 | SP1        | 9.501 | 0.870571327605649 | 230   | 239 | 1      | CTCGGCCTCC              |
| MA0079.1 | SP1        | 6.122 | 0.800408014237765 | 231   | 240 | -1     | TAGGGGCGGA               |
| MA0259.1 | ARNT::HIF1A| 6.391 | 0.856564524690194 | 266   | 273 | 1      | GACGCTG                 |
| MA0259.1 | ARNT::HIF1A| 4.936 | 0.813158318019073 | 281   | 288 | -1     | CTACGGGC                |
| MA0259.1 | ARNT::HIF1A| 8.854 | 0.930041835364402 | 306   | 313 | -1     | AACGCTG                 |
| MA0259.1 | ARNT::HIF1A| 8.575 | 0.92171858363548  | 316   | 323 | 1      | CCGGCTGC                |
| MA0079.1 | SP1        | 6.765 | 0.822836896268513 | 344   | 353 | 1      | CAGGGCCGTC               |
| MA0079.2 | SP1        | 7.611 | 0.822972926823903 | 345   | 354 | -1     | CACGCCCTC               |
| MA0079.3 | SP1        | 10.560 | 0.913997570755554 | 345   | 355 | -1     | GCACGGCCCCCT             |
| MA0079.1 | SP1        | 7.289 | 0.841114865450664 | 346   | 355 | 1      | GGCGGCTGC                |
| MA0079.1 | SP1        | 3.163 | 0.820934842377801 | 350   | 360 | -1     | CTACGGCACGG              |
| MA0079.2 | SP1        | 13.214 | 0.964080778453597 | 357   | 366 | -1     | CCCCCGCTCC               |
| MA0079.3 | SP1        | 13.420 | 0.949979644996513 | 357   | 367 | -1     | ACCCCGCCCTC              |
| MA0079.1 | SP1        | 11.046 | 0.972165113956893 | 358   | 367 | 1      | GACCGGCGGTC              |
| MA0079.3 | SP1        | 3.129 | 0.820507083453258 | 362   | 372 | -1     | CCCACACCCCG              |
| MA0079.2 | SP1        | 8.685 | 0.850020965965673 | 364   | 373 | -1     | CCCACACCCCG              |
| MA0079.3 | SP1        | 7.096 | 0.870416485031539 | 364   | 374 | -1     | GCCCCACACCC              |
| MA0079.2 | SP1        | 7.202 | 0.812672532157737 | 368   | 377 | -1     | CCCCCCACCAC              |
| MA0079.2 | SP1        | 8.757 | 0.851834179361406 | 369   | 378 | -1     | TCCCCGCCCTC              |
| MA0079.3 | SP1        | 10.509 | 0.91355932368739 | 369   | 379 | -1     | ATCCCCGGCCCT             |
| MA0079.1 | SP1        | 9.870 | 0.931144328158824 | 370   | 379 | 1      | GGGCGGGGAT               |
Table 2. Insulator sequences have been located on HRE sequence.

| Motif PWM | Motif Sequence                  | Input Sequence Name | Motif Start Location | Motif Length | Motif Orientation | Score     |
|-----------|--------------------------------|---------------------|----------------------|--------------|------------------|-----------|
| REN_20    | CCTACGGGACAGGGGACAC             | 1                   | 87                   | 20           | +                | -12.4488  |
| MIT_LM2   | GCGTGCCGCGGCGGGCCCAC           | 1                   | 153                  | 19           | +                | -13.4637  |
| MIT_LM2   | GCGTGCCGCGGCGGGCCCAC           | 1                   | 338                  | 19           | +                | -13.4637  |
| MIT_LM7   | GAGACAGCAGTGGGCAAAG             | 1                   | 189                  | 20           | +                | -7.411444 |
| MIT_LM23  | GAGACAGCAGTGGGCAAAG             | 1                   | 189                  | 20           | +                | -5.62159  |

Figure 4. A; ARNT consensus sequence has been repeated 9 times in HRE. B; sp1 consensus sequence has been repeated 26 times in HRE.

Figure 5. A; The results of immunofluorescence microscopy 48 hrs post transfection presented the specific expression of eGFP in the hypoxic condition at C2C12 myoblast cell line. eGFP was not detected in 4T1 cells in both hypoxic and normoxic condition. B; Flow cytometry results indicate the expression of eGFP under hypoxic condition in C2C12 cell line. C; Confirmation of hypoxia induction in both C2C12 and 4T1 cell lines by western blotting.

Expression cassette optimization
After subsequent transfection (48 h and 72 h), HREs exhibited the most intense fluorescence 48 hrs after transient transfection only in the hypoxic condition. Both cell lines were incubated under normoxic and hypoxic conditions to determine the function of HREs, including...
HIF-1α and sp1 transcription factor binding site. The expression vector generated a sharp eGFP expression, which was detected by immunofluorescence microscope 48 hrs after transfection in the C2C12 cell line under hypoxic conditions. On the other hand, No significant eGFP was detected in 4T1 cells. eGFP was expressed in C2C12 cell line but not 4T1 under hypoxic (O₂<1%) condition. Moreover, we cannot detect any significant eGFP signals in C2C12 under normoxia. Flow cytometry results showed that myoblast cells expressed eGFP in hypoxia. However, eGFP was not express in 4T1 cells in both hypoxia and normoxia. Overall, experiments demonstrated that our designed cassette was expressed and, hence, functioned appropriately (Figure 5A and B).

**eGFP measured 72h after transient transfection**

After confirmation of hypoxia inducible and tissue specific expressions in expression cassette, the green fluorescence signals were analyzed 72 h and 96 h after transient transfection. eGFP signals were detected after 72h of transient transfection by the immune fluorescence microscopy at 520 band pass filter. However, we could not detect eGFP signals after 96 h after transfection (Figure 6).

![Figure 6. eGFP measurement 72 h after transfection. Arrow indicated the eGFP expression in C2C12.](image)

**Discussion**

The results obtained through the experiments support some of our hypotheses. The current study explored the application of bioinformatics to improve the efficiency of cardiac gene therapy. Based on in silico analysis, we chose a cardiac specific cis-regulatory conserved motif that belonged to calsequestrin 2 (CASQ2) gene. The corresponding gene is specifically expressed in the heart. Tissue-specific cis-regulatory elements and correlated transcription factors, including myogenic regulatory factors (MRFs) like myogenic differentiation 1 (MYOD1), myogenic factor 5 (MYF5), myogenin and myogenin regulator factor 4 (MRF4), myogenic factor 6 (MYF6) interact with other transcription factors, particularly T box protein 2 (TBX2) and NK2 homeobox 5 (Nkx2.5), which play a pivotal role in the correct differentiation and progressive formation of cardiac muscle (Table 3). This summary helps us to understand the role of specific cis-regulatory elements in designing tissue-specific expression cassettes. Both CASQ2 and MLC2v have a specific transcription factor binding site (TFBSs), which is efficient for cardiac-specific transgene expression.

Rincon et al., 25 previously showed that CASQ2 had a strong specificity for cardiac muscle. They made a construct consisting of CASQ2 and myosin heavy chain α (MHCα) as a cardiac specific promoter. In this study, we used MLC2v because of its cardiac-specific regulatory elements (Table 4). For inducible expression under hypoxic conditions, we used HREs derived from phosphoglycerate kinase (PGK1), VEGF-165, and EPO promoter. However, it was shown that HRE was a conserved sequence itself, but the most noted HRE sequence was derived from the EPO promoter. 26 To make copies of inducible elements obtained from one promoter, overlap PCR must be performed. For easier construct production of the desired elements, we used promoter and 5’UTRs of three different genes, 27,28 HIF-1α belongs to a family of transcription factor with dimeric helices, containing basic amino acid residues that simplify DNA binding. bHLH proteins usually bind to a consensus sequence, well-known as HREs. Additionally, Sp1 is a zinc finger transcription factor that binds to GC-rich motifs of some promoters. 29,30 Sp1 binding sites are located near the HRE regions on the promoter. More studies have shown that sp1 protein becomes overexpressed under hypoxic conditions. 31 on the other side, hypoxic conditions have dual translation models known as cap-dependent and cap-independent (IRES) mechanisms. Notably, hypoxia has a strong restrictive effect on cap-dependent mRNA translation. 32 However, cellular IRES has a significant role in adaptation to hypoxic stress but is not increased at translational level, possibly due to inhibition of protein synthesis in order to conserve energy. IRESs are natural translational enhancers and, hence, mediate internal initiation of translation when present between desired genes.

In clinical assays, bicistronic IRES-based expression vectors have fewer side effects than monocistronic based expression vectors. When we designed a vector for hypoxia-inducible expression, strong viral IRESs were used for efficient cap-independent gene expression. Fundamentally, the IRES-based expression vector is efficient for in vitro and in vivo gene expression under hypoxic conditions. 33 Encephalomyocarditis virus (EMCV) is on-route into clinical studies and will hopefully be beneficial for patients. Our research, however, focused on in vitro studies. It is essential to study this cassette in vivo to evaluate all hypoxia-specific elements, such as enhancers (sp1, ARNT, and IRES) in a heterogeneous population of myoblast and adult myocytes. However, we demonstrated that these elements were efficient for gene expression on hypoxic myoblasts in an in vitro model.

**Conclusion**

In conclusion, combination of the cardiac/muscle specific cis-regulatory elements, CASQ2, and myosin
light chain-2 (MLC2v) have a significant specificity for myocytes. Both CASQ2 and MLC2v have a specific TFBS, which is efficient for cardiac-specific transgene expression. Sp1 binding sites are located near the HRE regions on the promoter. Therefore, Sp1 and HIF-1α have binding sites to a far distance of HREs derived from three promoters. Therefore, there is no longer need to overlap PCR process for one repeated sequence just in one promoter. This expression cassette well-designed for cardiac specific hypoxia inducible gene expression.

### Table 3. Cardiac specific transcription binding sites located on the CASQ2 enhancer.

| Model ID  | Model name | Score   | Relative score | Start | End | Strand | Predicted site sequence |
|-----------|------------|---------|----------------|-------|-----|--------|-------------------------|
| MA0037.2  | GATA3      | 3.279   | 0.820594050245556 | 7     | 14  | 1      | AGAAAAAC                |
| MA0052.2  | MEF2A      | 5.498   | 0.80187277397612  | 51    | 65  | 1      | TACCTTACATAGCTC         |
| MA0052.2  | MEF2A      | 8.618   | 0.84387374315264  | 90    | 104 | -1     | TCCTAAAATGGGTAGT        |
| MA0083.2  | SRF        | 8.230   | 0.817813133982383 | 90    | 107 | -1     | GCATCCTAAAATGGGTAGT     |
| MA0499.1  | Myod1      | 5.403   | 0.8596686986902211 | 105   | 117 | 1      | TCGAGTGTTGTCCA          |
| MA0052.2  | MEF2A      | 17.095  | 0.957989840107549 | 118   | 132 | 1      | GGCTAAAATAAATAC         |
| MA0063.1  | Nkx2-5     | 5.189   | 0.833795488710155 | 136   | 142 | -1     | TTCAATTG                |
| MA0035.3  | Gata1      | 2.402   | 0.800738582375976 | 151   | 161 | -1     | GTCGTATCTAA             |
| MA0037.2  | GATA3      | 6.588   | 0.872659931082488 | 153   | 160 | 1      | AGATACGA                |
| MA0482.1  | Gata4      | 2.124   | 0.802327146192479 | 176   | 186 | -1     | CTGTATCAGCG             |
| MA0499.1  | Myod1      | 0.838   | 0.800984257597511 | 191   | 203 | -1     | TCCACAGTGGGA            |
| MA0482.1  | Gata4      | 2.114   | 0.802183373083414 | 202   | 212 | -1     | TCTTCCCTCCTT            |
| MA0482.1  | Gata4      | 6.727   | 0.868505908295031 | 223   | 233 | -1     | TCTTGCTTTT              |
| MA0036.2  | GATA2      | 7.206   | 0.849841616420247 | 224   | 237 | -1     | ACATTCCTTGCTTT          |
| MA0035.3  | Gata1      | 6.525   | 0.857230665045272 | 224   | 234 | -1     | TCTTGCTTTT              |
| MA0037.2  | GATA3      | 6.588   | 0.872659931082488 | 226   | 233 | 1      | AGACAGA                 |
| MA0482.1  | Gata4      | 6.776   | 0.869210396529448 | 244   | 254 | -1     | CTTTATTTCAT             |
| MA0036.2  | GATA2      | 5.069   | 0.820706175169384 | 245   | 258 | -1     | GCTCTTCTATTTCA          |
| MA0035.3  | Gata1      | 5.099   | 0.837692049986718 | 245   | 255 | -1     | TCTTATTTTCA             |
| MA0037.2  | GATA3      | 5.281   | 0.852094773556387 | 247   | 254 | 1      | AAATAAGG                |
| MA0482.1  | Gata4      | 5.484   | 0.850634910838269 | 271   | 281 | -1     | TTTCCTCTCCT            |
| MA0035.3  | Gata1      | 2.802   | 0.806219259811756 | 272   | 282 | -1     | TTTCCTCTCCT             |
| MA0037.2  | GATA3      | 3.921   | 0.830695680797781 | 274   | 281 | 1      | AGAGAAAG                |
Table 4. Specific transcription factor and their binding sites are Myosin Light Chain 2 (MLC2).

| Entrez_ID | Symbol | Alias | GO_P | GO_C |
|-----------|--------|-------|------|------|
| 4250      | SCGB2A2| MGB1 UGB2 | na   | na   |
| 94234     | FOXQ1  | HFH1  | transcription; regulation of transcription, DNA dependent; hair follicle morphogenesis | nucleus |
| 6927      | TCF1   | HNF1 HNF1A LFB1 MODY3 | Bone resorption; positive regulation of transcription from RNA polymerase II promoter | nucleus; transcription factor complex |
| 6722      | SRF    | MCM1  | Heart looping; transcription; signal transduction; multicellular organisational development; Heart development; positive regulation of transcription from RNA polymerase II promoter; muscle maintenance | nucleus |
| 3170      | FOXA2  | HNF3B MGC19807 TCF3B | transcription; regulation of transcription, DNA dependent; lung development; epithelial cell differentiation; positive regulation of transcription from RNA polymerase II promoter; branching morphogenesis of a tube | nucleus |
| 3169      | FOXA1  | HNF3A MGC33105 TCF3A | transcription; regulation of transcription, DNA dependent; lung development; epithelial cell differentiation; hormone metabolic process; glucose homeostasis; positive regulation of transcription from RNA polymerase II promoter; branching morphogenesis of a tube | nucleus |
| 4763      | NF1    | DKFZp686J1293 NFNS VRNF WSS | cell cycle; Ras protein signal transduction; negative regulation of cell proliferation; regulation of glial cell differentiation; negative regulation of progression through cell cycle; regulation of small GTPase mediated signal transduction | intracellular; cytoplasm |

calsequestrin 2, bHLH: basic helix-loop-helix, PAS: PER-ARNT-SIM, EMCV: encephalomyocarditis virus, UTR: untranslated region, IRES: internal ribosome entry site, MRFs: myogenic regulatory factors, MYOD1: myogenic differentiation 1, MYF5: myogenic factor 5, MYF6: myogenic factor 6, MHC-α: myosin heavy chain- alpha, TBX2: T box protein 2, NK2-5: NK2 homeobox 5.

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Ethical Issues
Not applicable.

Conflict of Interest
The authors declare “no” conflict of interest.

Abbreviations
HREs: hypoxia response elements, HIF-1α: hypoxia inducible factor-1 alpha, VEGF-A: vascular endothelial growth factor-A, PGK-1: phosphoglycerate kinase 1, MLC2: myosin light chain, EPO: erythropoietin, ARNT: aryl hydrocarbon receptor nuclear translocator, EGFP: enhanced green fluorescent protein, CASQ2: calsequestrin 2, bHLH: basic helix-loop-helix, PAS: PER-ARNT-SIM, EMCV: encephalomyocarditis virus, UTR: untranslated region, IRES: internal ribosome entry site, MRFs: myogenic regulatory factors, MYOD1: myogenic differentiation 1, MYF5: myogenic factor 5, MYF6: myogenic factor 6, MHC-α: myosin heavy chain- alpha, TBX2: T box protein 2, NK2-5: NK2 homeobox 5.

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