Published in final edited form as:

J Cardiovasc Transl Res. 2009 June ; 2(2): 182–190. doi:10.1007/s12265-009-9089-6.

Chronic Treatment with Clenbuterol Modulates Endothelial Progenitor Cells and Circulating Factors in a Murine Model of Cardiomyopathy

James E. Rider,

Lillehei Heart Institute, University of Minnesota, 312 Church Street, Minneapolis, MN 55455, USA

Sean P. Polster,

Lillehei Heart Institute, University of Minnesota, 312 Church Street, Minneapolis, MN 55455, USA

Sangjin Lee,

Lillehei Heart Institute, University of Minnesota, 312 Church Street, Minneapolis, MN 55455, USA

Nathan J. Charles,

Lillehei Heart Institute, University of Minnesota, 312 Church Street, Minneapolis, MN 55455, USA

Neeta Adhikari,

Lillehei Heart Institute, University of Minnesota, 312 Church Street, Minneapolis, MN 55455, USA

Ami Mariash,

Lillehei Heart Institute, University of Minnesota, 312 Church Street, Minneapolis, MN 55455, USA

George Tadros,

Lillehei Heart Institute, University of Minnesota, 312 Church Street, Minneapolis, MN 55455, USA

Jenna Stangland,

Lillehei Heart Institute, University of Minnesota, 312 Church Street, Minneapolis, MN 55455, USA

Ryszard T. Smolenski,

Heart Science Centre, National Heart and Lung Institute, Imperial College London, Harefield, Middlesex, UK

Cesare M Terracciano,

Heart Science Centre, National Heart and Lung Institute, Imperial College London, Harefield, Middlesex, UK

Paul J.R. Barton,

Heart Science Centre, National Heart and Lung Institute, Imperial College London, Harefield, Middlesex, UK

Emma J. Birks,

Heart Science Centre, National Heart and Lung Institute, Imperial College London, Harefield, Middlesex, UK

Magdi H. Yacoub,

Heart Science Centre, National Heart and Lung Institute, Imperial College London, Harefield, Middlesex, UK

Leslie W. Miller, and

Washington Hospital Center and Georgetown University, Washington, DC 20010, USA

Jennifer L. Hall

Lillehei Heart Institute, University of Minnesota, 312 Church Street, Minneapolis, MN 55455, USA

Jennifer L. Hall: jlhall@umn.edu

Abstract

The purpose of this study was to determine the effects of chronic treatment with the beta 2 adrenergic receptor agonist clenbuterol on endothelial progenitor cells (EPC) in a well-characterized model of heart failure, the muscle LIM protein knockout (MLP^{-/-}) mouse. MLP^{-/-} mice were treated daily with clenbuterol (2 mg/kg) or saline subcutaneously for 6 weeks. Clenbuterol led to a 30% increase in CD31⁺ cells in the bone marrow of MLP^{-/-} heart failure mice ($p < 0.004$). Clenbuterol did not improve ejection fraction. Clenbuterol treatment in MLP^{-/-} mice was associated with significant changes in the following circulating factors: tissue inhibitor of metalloproteinase-type 1, leukemia inhibitory factor 1, C-reactive protein, apolipoprotein A1, fibroblast growth factor 2, serum glutamic oxaloacetic transaminase, macrophage-derived chemokine, and monocyte chemoattractant protein-3. Clen-butanol treatment in the MLP^{-/-} model of heart failure did not rescue heart function, yet did increase CD31⁺ cells in the bone marrow. This is the first evidence that a beta 2 agonist increases EPC proliferation in the bone marrow in a preclinical model of heart failure.

Keywords

Clenbuterol; Heart Failure; Muscle LIM Protein; Beta 2 Adrenergic Receptor; Endothelial Progenitor Cell

Introduction

Recent findings suggest a new role for the sympathetic nervous system in the proliferation and mobilization of progenitor cells that may be related to cardiovascular disease. In brief, the number of progenitor cells released from the bone marrow into the peripheral blood increases under conditions of stress, inflammation, and tissue repair [7]. Proliferation of progenitor cells in the bone marrow is considered a prerequisite for mobilization [43]. Evidence is mounting that this process becomes increasingly dysfunctional in subjects with inherent cardiovascular risk factors as well as subjects with congestive heart failure [14, 20, 29, 36, 38, 40]. The number of circulating EPC is reduced in subjects with increased risk of cardiovascular disease and patients with heart failure [14, 29]. Recent preclinical work has shown that the beta adrenergic agonists clenbuterol and epinephrine increase the proliferation and the mobilization of progenitor cells in healthy mice [19, 32]. The non-specific beta blocker, propranolol, diminished these effects [19, 32]. These findings open up

new windows into the potential pharmacological effects of beta adrenergic agonists and antagonists in patients with heart failure.

The potential role of clenbuterol, a beta 2 adrenergic agonist, on EPC in the bone marrow and circulation in a model of heart failure has not been described. Thus, the first goal of this study was to determine the effects of clenbuterol on EPC populations in the bone marrow and circulation in the setting of heart failure.

Preclinical and clinical studies have demonstrated that clenbuterol induces hypertrophy in skeletal and cardiac muscle in association with marked changes in gene expression, excitation contraction coupling, and cellular metabolism [5, 11, 12, 27, 30, 31, 41, 42]. Significant improvements in ventricular function were also reported with a combination therapy that included the beta 2 adrenergic agonist clenbuterol in a subset of heart failure patients supported with a mechanical ventricular assist device [6]. However, the molecular mechanisms responsible for these improvements in function were not clear. Given that changes have been seen in vivo in skeletal and cardiac muscle, we hypothesize that clenbuterol may be altering the circulating profile of cytokines and growth factors.

A well-characterized transgenic model of chronic heart failure, the muscle LIM protein knockout (MLP^{-/-}) mouse, was chosen as it closely resembles many attributes of dilated heart failure including dysregulated beta adrenergic signaling, abnormalities in the cytoskeletal proteins, morphological changes, impaired calcium handling, and depressed cardiovascular function [1–3, 8, 13, 15, 16, 22, 26, 33, 37]. Furthermore, this model eliminates nonspecific inflammatory effects from a surgery or intervention.

We provide evidence that 6 weeks of treatment with clenbuterol resulted in a 30% increase in CD31⁺ cells in the bone marrow of MLP^{-/-} mice. This was accompanied by marked changes in circulating levels of apolipoprotein A1 (Apo-A1), C-reactive protein (CRP), fibroblast growth factor 2 (FGF-2), leukemia inhibitory factor 1 (LIF-1), monocyte chemoattractant protein-3 (MCP-3), macrophage-derived chemokine (MDC), serum glutamic oxaloacetic transaminase (SGOT), and tissue inhibitor of metalloproteinase-type 1 (TIMP-1). Ejection fractions in the clenbuterol-treated MLP^{-/-} model of heart failure were not different from saline-treated MLP^{-/-} mice at 1 week but were significantly decreased at 6 weeks. These data suggest that clenbuterol is not sufficient to rescue heart failure in a mouse model with impaired beta adrenergic signaling. However, it does increase the number of CD31⁺ cells in the bone marrow.

Materials and Methods

Study Design

Mice were housed in a pathogen-free facility and handled in accordance with standard use protocols, animal welfare regulations, and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All protocols were approved by the University of Minnesota Institutional Animal Care and Use Committee. One-year-old male MLP^{-/-} mice were injected subcutaneously (s.c.) with saline (n=3) or clenbuterol (2 mg/kg, n=4) daily for 6 weeks. These animals were studied for cardiovascular function at 1 and 6 weeks and for

the circulating serum factors analysis at 6 weeks. We saw no function improvement in this cohort. Thus in a second study we administered clenbuterol to a cohort of three month old MLP^{-/-} mice (compared to the 1 year old mice in the first cohort). Three-month-old male MLP^{-/-} mice (129Sv/Swiss/C57BL6 mixed background) were injected subcutaneously (s.c.) with saline (n=9) or clenbuterol (2 mg/kg; n=10) daily for 6 weeks. All animals were weighed daily and the dose of clenbuterol adjusted accordingly. Animals were euthanized at 6 weeks, and hearts were weighed and snap-frozen in liquid nitrogen. Bone marrow was harvested, and blood was obtained via ventricular puncture and preserved with ethylenediamine tetraacetic acid at the time the animals were euthanized. Age-matched C57BL6 wild-type (WT) animals underwent no treatment (n=6) and were used for comparison purposes for real-time quantitative polymerase chain reaction (RT-PCR) and fluorescence-activated cell sorting (FACS).

Real-Time Quantitative PCR

RNA was isolated from tissue using the RNeasy Mini Kit according to the manufacturers directions (Qiagen, Valencia, CA, USA). RNA yield and purity was assessed by ratio optical density at 260 and 280 nm. Using random hexamer primers, 1 µg of total RNA was reverse-transcribed using the Advantage RT-for-PCR Kit (BD Biosciences Clontech, Mountain View, CA, USA) as we have previously described [39]. The following primer sequences were used for RT-PCR reactions all with annealing temperatures of 58°C : BNP Forward 5'-CAGC TCTTGAAGGACCAAGG-3' BNP Reverse 5'-GACCCAGG CAGAGTCAGAA-3' ADRB1 Forward 5'-GTAACGTG CTGGTGATCGTG-3' ADRB1 Reverse 5'-AAGTCCA GAGCTCGCAGAAG-3' ADRB2 Forward 5'-AAAGAGCA CAAAGCCCTCAA-3' ADRB2 Reverse 5'-GCTCTTGA AAGGCAATCCTG-3' TIMP-1 Forward 5'-TTCAAG GCTGTGGGAAATG-3' TIMP-1 Reverse 5'-CTCAGAG TACGCCAGGGAAC-3'. Reactions using these primers were carried out using the LightCycler FastStart DNA Master SYBR Green I Kit (Roche Diagnostics, Penzberg, Germany) on the Roche Light cycler and normalized to 18S [39]. 18S expression was not statistically different between cohorts. Amplicon products were run on a gel to confirm size. Expression is shown as target gene/18S expression. Additional studies were carried out using Applied Biosystems (Foster City, CA) 7900HT Fast RealTime PCR system. Taq-Man primer-probe pairs were ordered from Applied Biosystems for the following genes: Beta 1-adrenergic receptor (ADRB1 Mm00431701_s1), beta 2-adrenergic receptor (ADRB2 Mm_02524224_s1), Cyclo-philin A (Ppia Mm_02342429_g1), and hypoxanthine-guanine phosphoribosyl transferase (HPRT1 Mm_01545399_m1). The Taqman reactions were run with an annealing temperature of 60°C. The levels of Cyclophilin A or HPRT1 on each sample were used to normalize for variability in RNA quality or in the efficiency of the reverse transcriptase reaction as we have previously described [39].

Expression of HPRT1 and Cyclophilin were unchanged between cohorts. Gene expression was calculated using the Ct method. The efficiency of all reactions was quantified as previously described [10].

Echocardiography

Transthoracic echocardiograms were obtained using a Vevo 770 High-resolution Imaging System (VisualSonics, Toronto, Canada) with a 30 MHz imaging transducer. MLP^{-/-} mice were anesthetized with isoflurane for imaging. Echocardiograms were analyzed separately by two cardiologists blinded to the study design.

Serum Analysis

The serum concentration of a panel of circulating factors was measured in the study utilizing Luminex Multi-Analyte Profiles on the 1-year-old cohort (n=4 MLP^{-/-} clenbuterol, n=3 MLP^{-/-} saline; Rules Based Medicine, Austin, TX, USA).

Clenbuterol Levels

Clenbuterol levels in the blood of MLP^{-/-} (treated with saline or clenbuterol) were measured with high-performance liquid chromatography as previously described 22 h post final injection (n=6 for each group) [44].

Fluorescence-Activated Cell Characterization and Sorting

Bone marrow (femur) and peripheral blood were isolated from 1-year-old MLP^{-/-} (treated with saline or clenbuterol) or WT (C57BL6) mice. Lysis of red blood cells was performed using ACK lysis buffer. Cells were counted with trypan blue to ensure equal numbers of cells in each sample. Cells (1×10^6) were labeled directly with mouse IgG anti-CD45-PerCP, anti-CD34-PE, or anti-CD31-APC (Becton Dickinson, Franklin Lakes, NJ, USA). Isotype specific antibodies were used to establish background fluorescence. The sorting population was prepared by triple staining 8×10^7 cells. The labeled cells were analyzed by three-color flow cytometry with a FACSDivide cytometer (Becton Dickinson). Viable cells were identified by gating on forward/side scatter.

Statistical Analysis

To assess statistical difference between means, the Student's *t* test was used. $p < 0.05$ was considered significant. Results are expressed as means $\pm$ standard error unless otherwise noted. Assaying statistical difference between more than two groups was done with a one-way analysis of variance done in the statistical software suite SigmaStat. "n" refers to the number of samples in each group for the specified study. Pearson product-moment correlation coefficient test was used to assess relationships between two groups.

Results

Clenbuterol (2 mg/kg s.c.) or saline was administered daily for 6 weeks in the MLP^{-/-} mouse. High-performance liquid chromatography analyses demonstrated that this dose led to a significant elevation in circulating clenbuterol ($0.0182 \mu\text{M} \pm 0.003$ in clenbuterol-treated samples vs. 0.000 ± 0.000 in saline controls, $n=6$, $p < 0.001$). This level is within the therapeutic range for the treatment of heart failure patients [44].

We assessed CD31⁺ and CD34⁺ cells in the bone marrow of WT and MLP^{-/-} heart failure mice in the presence and absence of clenbuterol with fluorescence-activated cell sorting.

Clenbuterol increased CD31⁺ cells in the bone marrow of MLP^{-/-} mice by 30% ($p < 0.004$, Fig. 1). Clenbuterol had no effect on CD34⁺ cells in the bone marrow. CD31⁺ and CD34⁺ cells in the circulation were not different between clenbuterol-treated MLP^{-/-} and saline MLP^{-/-} (data not shown).

Clenbuterol treatment in MLP^{-/-} mice was associated with a decline in left ventricular ejection fraction compared to saline-treated MLP^{-/-} at week 6 (Table 1). This finding was similar to results in the 1 year old cohort of clenbuterol-treated MLP^{-/-} mice ($n=4$; clenbuterol, $21.0 \pm 0.6\%$ vs. saline $n=3$, $28.8 \pm 2.9\%$, $p < 0.003$). In this 1-year-old cohort of mice, echocardiograms were also performed after 1 week of clenbuterol treatment. Clenbuterol did not alter ejection fraction in the MLP^{-/-} mice at this time point (week 1; clenbuterol, $28.8 \pm 3.7\%$ vs. saline, $28.0 \pm 4.8\%$, $p = \text{ns}$).

We analyzed circulating factors in the serum 6 weeks after clenbuterol or saline treatment in the MLP^{-/-} mice (Table 2). Clenbuterol treatment was associated with marked changes in circulating levels of Apo-A1, CRP, FGF-2, LIF-1, MCP-3, MDC, SGOT, and TIMP-1 protein. TIMP-1 expression in the serum increased with clenbuterol treatment (0.6 ± 0.1 vs. 1.6 ± 0.3 ng/ml, $p < 0.044$). TIMP-1 has been implicated with adverse cardiac remodeling during failure [4]. TIMP-1 mRNA expression in the heart correlated strongly with BNP mRNA expression ($r = 0.81$, Fig. 2). BNP mRNA levels in the heart were 0.06 ± 0.02 WT (C57BL6; $n=5$), 0.61 ± 0.13 MLP^{-/-} saline ($n=4$), and 0.85 ± 0.17 MLP^{-/-} clenbuterol ($n=5$; WT vs. saline MLP^{-/-}, $p < 0.05$; WT vs. clenbuterol MLP^{-/-}, $p < 0.05$; clenbuterol MLP^{-/-} vs. saline MLP^{-/-}, $p = \text{ns}$).

LIF-1 protein was also elevated in the serum of clenbuterol-treated MLP^{-/-} heart failure mice (163 ± 2.7 vs. 247 ± 15.3 ; $p < 0.0059$; Table 2). The inverse correlation between LIF-1 protein and left ventricular ejection fractions ($r = 0.85$, Fig. 3) suggests that the increase in LIF-1 protein may have been due to a worsening ejection fraction and not a direct effect of clenbuterol in the heart. This would lead which would confirm previously published work [18, 35, 45].

Expression levels of beta 1-adrenergic receptor mRNA and beta 2-adrenergic receptor mRNA were assayed in heart tissue and bone marrow in the MLP^{-/-} model of heart failure and WT controls. Beta 1-adrenergic receptor mRNA expression was decreased in hearts of MLP^{-/-} mice compared to WT controls (Fig. 4) ($p < 0.01$). Beta 2-adrenergic receptor mRNA was decreased in hearts of MLP^{-/-} mice treated with clenbuterol compared to WT controls (Fig. 4) ($p < 0.03$). This work extends earlier work by Rockman et al., showing a decline in beta adrenergic receptor density in hearts of MLP^{-/-} mice compared to WT controls [9]. Neither beta 1-adrenergic receptor nor beta 2-adrenergic receptor mRNA was decreased in the bone marrow of MLP^{-/-} mice compared to WT controls (Fig. 5). Clenbuterol had no effect on either beta 1-adrenergic receptor mRNA or beta 2-adrenergic receptor mRNA expression in the bone marrow of MLP^{-/-} mice (Fig. 5).

Discussion

We provide evidence that chronic clenbuterol treatment was associated with an increase in CD31⁺ EPC in the bone marrow of the MLP^{-/-} model of heart failure. The second major finding was that chronic treatment with clenbuterol was associated with a decline in ejection fraction in this MLP^{-/-} model. Finally, clenbuterol was associated with significant changes in the circulating protein levels of Apo-A1, CRP, FGF-2, LIF-1, MCP-3, MDC, SGOT, and TIMP-1.

This MLP^{-/-} model has impaired beta adrenergic signaling [28]. Clenbuterol treatment in the MLP^{-/-} mouse led to a decline in cardiovascular function. These data support earlier work by Rockman et al. showing a similar functional decline in the MLP^{-/-} mouse when crossed with a mouse harboring cardiac-specific overexpression of the beta 2-adrenergic receptor [28]. The MLP^{-/-} dilated heart phenotype can be reversed by deleting phospholamban [25]. In the MLP^{-/-} model, diastolic calcium extrusion is abnormally high in association with increased sarco/ endoplasmic reticulum Ca²⁺-ATPase activity and increased phosphorylation of phospholamban [1, 33]. If clenbuterol is acting directly at the level of excitation contraction coupling, one might predict worsening left ventricular function in the MLP^{-/-} model given that clenbuterol would increase sarcoplasm reticulum calcium uptake, reduce contractive reserves and worsen LV function. Our findings support this view. Finally, our previous work would suggest that clenbuterol may also act at the level of the sarcomere leading to changes in the transcripts of sarcomeric proteins including alpha1-actinin in human cardiomyopathy [5, 12]. MLP interacts with alpha1-actinin at the Z disc [21]. Loss of MLP has been shown to lead to disruption of the Z disc and passive stretch properties of isolated papillary muscles. Thus, if clenbuterol is acting at the level of the sarcomere and requires an intact Z disc to improve diastolic function, one might again predict that no improvements would be seen in this model.

Preclinical work by Spiegel et al. demonstrated that epinephrine induced significant increases in progenitor cell proliferation and migration in healthy mice [32]. This group also showed that treatment with the nonspecific beta adrenergic antagonist, propranolol, reduced the progenitor cell number in the bone marrow. In addition, Katayama et al. showed that clenbuterol in combination with granulocyte-colony stimulation factor stimulates progenitor cell mobilization in healthy mice [19]. Progenitor cell proliferation in the bone marrow was not measured in this study. The work presented in this paper demonstrates that chronic clenbuterol treatment is able to stimulate EPC proliferation in a murine model of heart failure. This is a novel finding. Previous work in the field suggests that stimulation with clenbuterol alone may not be sufficient for progenitor cell mobilization [19]. Our work confirms this finding. Perhaps one of the most important unanswered questions in the field is how progenitor cell proliferation and mobilization may be contributing to cardiovascular benefit or risk. Previous work implicates the Wnt pathway in progenitor cell mobilization and signaling [32]. Future work will be needed to explore the role of Wnt signaling and other potential mechanisms in mediating the effect of clenbuterol on CD31⁺ cells in the bone marrow.

Clenbuterol's impact on circulating factors in the serum was assessed with Luminex bead based multiplexing technology (Table 2). We followed up on TIMP-1 given its potential role in remodeling [24]. In the Framingham heart study, TIMP-1 levels correlated with other cardiovascular risk factors and cardiovascular disease itself [34]. We see similar findings in this study. However, we also report an association with CD31⁺ in the bone marrow and TIMP-1 in the circulation (Pearson correlation coefficient of 0.68). This should be interpreted with caution given the small sample size (n=8). The increase in circulating TIMP-1 protein may be an important component of the systemic effects of clenbuterol in the bone marrow. This will warrant future study as TIMP-1 may be a prognostic indicator of cardiovascular risk as well as a response to the bone marrow population modulation.

Clenbuterol reduced the circulating protein levels of SGOT by half. SGOT is a well-known marker of generalized muscle damage [23], and clenbuterol has been shown to reduce plasma levels of SGOT in rats with endotoxin-induced liver damage [17]. The effect of clenbuterol treatment on other circulating factors including the increase in FGF-2 and decrease in CRP in this model of heart failure will also require further study. CRP in the cohort treated with clenbuterol was below the detection threshold (0.50 µg/ml), but the trend maintains a decrease between the groups. All other measurements are well above the minimal level of detection. It is unclear how clenbuterol may be altering Apo-A1, MDC, and MCP-3 levels.

There are several limitations of this study. First, it would have been advantageous to determine the effect of clenbuterol on additional subpopulations of cells in the bone marrow and the circulation. This will require future studies. Secondly, more data will need to be collected to suggest a mechanism involving clenbuterol and CD31⁺ cell proliferation in the marrow and why changes are not seen in the circulating profile. Receptor expression levels of ADRB1 and ADRB2 were explored in different cell subtypes in an attempt to further understand the mechanism behind the CD31⁺ bone marrow increase. CD31⁺ and CD34⁺ cell types both harbor ADRB1 and ADRB2, but we saw no difference in regulation in the chronic setting of heart failure. We recognize that measuring bone marrow cell populations and cells in the circulation do not account for the continual changes in cell turnover in the bone marrow, apoptosis in the circulation and bone marrow, and/or uptake and incorporation of progenitor cells by tissues. Thus, tracking progenitor cell biology and the role of beta agonists and beta blockers presents a difficult challenge that will require new tools and approaches.

It would be particularly advantageous to have bone marrow, circulating cell, and serum measurements at the earlier time points when there were no significant changes in ventricular function. Despite these limitations, demonstrating that clenbuterol treatment is associated with a significant increase in the number of CD31⁺ cells is a first step toward understanding the impact of clenbuterol in the bone marrow in preclinical models. These findings shed new light on progenitor cell biology in response to beta 2 agonists in preclinical models of heart failure.

In conclusion, we provide evidence that chronic treatment with clenbuterol is associated with a significant increase in the number of CD31⁺ cells in the bone marrow in a model of

heart failure. Finally, we demonstrate that clenbuterol is associated with marked changes in the circulating profile of growth factors and chemokines in this preclinical model of heart failure. A current limitation of these findings is that we are unable to determine if the increase in CD31⁺ cells in the bone marrow and/or the changes in circulating factors are due to clenbuterol alone, and/or the alterations in heart failure function. Future studies will be needed to address this.

Acknowledgments

The authors wish to thank the staff at Luminex Multi-Analyte Profiles (Rules Based Medicine, Austin, Texas).

References

1. Antoons G, Vangheluwe P, Volders PG, Bito V, Holemans P, Ceci M, Wuytack F, Caroni P, Mubagwa K, Sipido KR. Increased phospholamban phosphorylation limits the force-frequency response in the MLP^{-/-} mouse with heart failure. *Journal of Molecular and Cellular Cardiology*. 2006; 40:350–360. [PubMed: 16427649]
2. Arber S, Hunter JJ, Ross J, Hongo M, Sansig G, Borg J, Perriard JC, Chien KR, Caroni P Jr. MLP-deficient mice exhibit a disruption of cardiac cytoarchitectural organization, dilated cardiomyopathy, and heart failure. *Cell*. 1997; 88:393–403. [PubMed: 9039266]
3. Badea CT, Hedlund LW, Mackel JF, Mao L, Rockman HA, Johnson GA. Cardiac micro-computed tomography for morphological and functional phenotyping of muscle LIM protein null mice. *Molecular Imaging*. 2007; 6:261–268. [PubMed: 17711781]
4. Barton PJBE, Felkin LE, Cullen ME, Koban MU, Yacoub MH. Increased expression of extracellular matrix regulators TIMP1 and MMP1 in deteriorating heart failure. *Journal of Heart and Lung Transplantation*. 2003; 22:738–744. [PubMed: 12873541]
5. Birks EJ, Hall JL, Barton PJ, Grindle S, Latif N, Hardy JP, Rider JE, Banner NR, Khaghani A, Miller LW, Yacoub MH. Gene profiling changes in cytoskeletal proteins during clinical recovery after left ventricular-assist device support. *Circulation*. 2005; 112:157–164. [PubMed: 16159866]
6. Birks EJ, Tansley PD, Hardy J, George RS, Bowles CT, Burke M, Banner NR, Khaghani A, Yacoub MH. Left ventricular assist device and drug therapy for the reversal of heart failure. *The New England Journal of Medicine*. 2006; 355:1873–1884. [PubMed: 17079761]
7. Cottler-Fox MH, Lapidot T, Petit I, Kollet O, DiPersio JF, Link D, Devine S. Stem cell mobilization. *Hematology*. 2003; 2003:419–437. [PubMed: 14633793]
8. Ehler E, Horowitz R, Zuppinger C, Price RL, Perriard E, Leu M, Caroni P, Sussman M, Eppenberger HM, Perriard JC. Alterations at the intercalated disk associated with the absence of muscle LIM protein. *The Journal of Cell Biology*. 2001; 153:763–772. [PubMed: 11352937]
9. Esposito G, Santana LF, Dilly K, Cruz JD, Mao L, Lederer WJ, Rockman HA. Cellular and functional defects in a mouse model of heart failure. *American Journal of Physiology Heart and Circulatory Physiology*. 2000; 279:H3101–H3112. [PubMed: 11087268]
10. Fleige S, Walf V, Huch S, Prgomet C, Sehm J, Pfaffl MW. Comparison of relative mRNA quantification models and the impact of RNA integrity in quantitative real-time RT-PCR. *Biotechnology Letters*. 2006; 28:1601–1613. [PubMed: 16900335]
11. George I, Xydas S, Mancini DM, Lamanca J, DiTullio M, Marboe CC, Shane E, Schulman AR, Colley PM, Petrilli CM, Naka Y, Oz MC, Maybaum S. Effect of clenbuterol on cardiac and skeletal muscle function during left ventricular assist device support. *Journal of Heart and Lung Transplantation*. 2006; 25:1084–1090. [PubMed: 16962470]
12. Hall JL, Birks EJ, Grindle S, Cullen ME, Barton PJ, Rider JE, Lee S, Harwalker S, Mariash A, Adhikari N, Charles NJ, Felkin LE, Polster S, George RS, Miller LW, Yacoub MH. Molecular signature of recovery following combination left ventricular assist device (LVAD) support and pharmacologic therapy. *European Heart Journal*. 2007; 28:613–627. [PubMed: 17132651]
13. Heineke J, Ruetten H, Willenbockel C, Gross SC, Naguib M, Schaefer A, Kempf T, Hilfiker-Kleiner D, Caroni P, Kraft T, Kaiser RA, Molkenin JD, Drexler H, Wollert KC. Attenuation of

cardiac remodeling after myocardial infarction by muscle LIM protein-calcineurin signaling at the sarcomeric Z-disc. *Proceedings of the National Academy of Sciences of the United States of America*. 2005; 102:1655–1660. [PubMed: 15665106]

14. Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, Finkel T. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *The New England Journal of Medicine*. 2003; 348:593–600. [PubMed: 12584367]
15. Hoit BD. Excitation-contraction coupling in the MLP knockout mouse. *Journal of Molecular and Cellular Cardiology*. 2006; 40:335–338. [PubMed: 16460751]
16. Hongo M, Ryoike T, Schoenfeld J, Hunter J, Dalton N, Clark R, Lowe D, Chien K, Ross J Jr. Effects of growth hormone on cardiac dysfunction and gene expression in genetic murine dilated cardiomyopathy. *Basic Research in Cardiology*. 2000; 95:431–441. [PubMed: 11192363]
17. Izeboud CAHK, Grootendorst AF, Nijmeijer SM, van Miert AS, Witkamp RR, Rodenburg RJ. Endotoxin-induced liver damage in rats is minimized by beta 2-adrenoceptor stimulation. *Inflammation Research*. 2004; 53:93–99. [PubMed: 15021963]
18. Jougasaki M, Leskinen H, Larsen AM, Cataliotti A, Chen HH, Burnett JC Jr. Leukemia inhibitory factor is augmented in the heart in experimental heart failure. *European Journal of Heart Failure*. 2003; 5:137–145. [PubMed: 12644003]
19. Katayama Y, Battista M, Kao WM, Hidalgo A, Peired AJ, Thomas SA, Frenette PS. Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. *Cell*. 2006; 124:407–421. [PubMed: 16439213]
20. Kissel CK, Lehmann R, Assmus B, Aicher A, Honold J, Fischer-Rasokat U, Heeschen C, Spyridopoulos I, Dimmeler S, Zeiher AM. Selective functional exhaustion of hematopoietic progenitor cells in the bone marrow of patients with postinfarction heart failure. *Journal of the American College of Cardiology*. 2007; 49:2341–2349. [PubMed: 17572250]
21. Knoll R, Hoshijima M, Hoffman HM, Person V, Lorenzen-Schmidt I, Bang ML, Hayashi T, Shiga N, Yasukawa H, Schaper W, McKenna W, Yokoyama M, Schork NJ, Omens JH, McCulloch AD, Kimura A, Gregorio CC, Poller W, Schaper J, Schultheiss HP, Chien KR. The cardiac mechanical stretch sensor machinery involves a Z disc complex that is defective in a subset of human dilated cardiomyopathy. *Cell*. 2002; 111:943–955. [PubMed: 12507422]
22. Lorenzen-Schmidt I, Stuyvers BD, ter Keurs HE, Date MO, Hoshijima M, Chien KR, McCulloch AD, Omens JH. Young MLP deficient mice show diastolic dysfunction before the onset of dilated cardiomyopathy. *Journal of Molecular and Cellular Cardiology*. 2005; 39:241–250. [PubMed: 15978612]
23. Panteghini M. Aspartate aminotransferase isoenzymes. *Clinical Biochemistry*. 1990; 23:311–319. [PubMed: 2225456]
24. McKie PMBJJ. B-type natriuretic peptide as a biomarker beyond heart failure: speculations and opportunities. *Mayo Clinic Proceedings*. 2005; 80:1029–1036. [PubMed: 16092582]
25. Minamisawa S, Hoshijima M, Chu G, Ward CA, Frank K, Gu Y, Martone ME, Wang Y, Ross J, Kranias EG, Giles WR, Chien KR Jr. Chronic phospholamban-sarcoplasmic reticulum calcium ATPase interaction is the critical calcium cycling defect in dilated cardiomyopathy. *Cell*. 1999; 99:313–322. [PubMed: 10555147]
26. Omens JH, Usyk TP, Li Z, McCulloch AD. Muscle LIM protein deficiency leads to alterations in passive ventricular mechanics. *American Journal of Physiology*. 2002; 282:H680–H687. [PubMed: 11788418]
27. Petrou M, Wynne DG, Boheler KR, Yacoub MH. Clenbuterol induces hypertrophy of the latissimus dorsi muscle and heart in the rat with molecular and phenotypic changes. *Circulation*. 1995; 92:II483–II489. [PubMed: 7586459]
28. Rockman HACK, Choi DJ, Iaccarino G, Hunter JJ, Ross J, Lefkowitz RJ, Koch WJ Jr. Expression of a beta-adrenergic receptor kinase 1 inhibitor prevents the development of myocardial failure in gene-targeted mice. *Proceedings of the National Academy of Sciences of the United States of America*. 1998; 95:7000–7005. [PubMed: 9618528]
29. Schmidt-Lucke C, Rossig L, Fichtlscherer S, Vasa M, Britten M, Kamper U, Dimmeler S, Zeiher AM. Reduced number of circulating endothelial progenitor cells predicts future cardiovascular

- events: proof of concept for the clinical importance of endogenous vascular repair. *Circulation*. 2005; 111:2981–2987. [PubMed: 15927972]
30. Soppa GK, Lee J, Stagg MA, Felkin LE, Barton PJ, Siedlecka U, Youssef S, Yacoub MH, Terracciano CM. Role and possible mechanisms of clenbuterol in enhancing reverse remodelling during mechanical unloading in murine heart failure. *Cardiovascular Research*. 2008; 77:695–706. [PubMed: 18178572]
 31. Soppa GKSR, Latif N, Yuen AH, Malik A, Karbowska J, Kochan Z, Terracciano CM, Yacoub MH. Effects of chronic administration of clenbuterol on function and metabolism of adult rat cardiac muscle. *American Journal of Physiology*. 2005; 288:H1468–H1476. [PubMed: 15528231]
 32. Spiegel A, Shvitiel S, Alexander K, Aya L, Neta N, Polina G, Yaara A, Igor R, Hardan I, Herzel B, Arnon N, Menachem R, Tsvee L. Catecholaminergic neurotransmitters regulate migration and repopulation of immature human CD34+ cells through Wnt signaling. *Nature Immunology*. 2007; 8:1123–1131. [PubMed: 17828268]
 33. Su Z, Yao A, Zubair I, Sugishita K, Ritter M, Li F, Hunter JJ, Chien KR, Barry WH. Effects of deletion of muscle LIM protein on myocyte function. *American Journal of Physiology*. 2001; 280:H2665–H2673. [PubMed: 11356623]
 34. Sundstrom JEJ, Benjamin EJ, Levy D, Larson MG, Sawyer DB, Siwik DA, Colucci WS, Wilson PWF, Vasan RS. Relations of plasma total TIMP-1 levels to cardiovascular risk factors and echocardiographic measures: the Framingham heart study. *European Heart Journal*. 2004; 25:1509–1516. [PubMed: 15342170]
 35. Tsuruda T, Boerrigter G, Huntley BK, Noser JA, Cataliotti A, Costello-Boerrigter LC, Chen HH, Burnett JC Jr. Brain natriuretic Peptide is produced in cardiac fibroblasts and induces matrix metalloproteinases. *Circulation Research*. 2002; 91:1127–1134. [PubMed: 12480813]
 36. Valgimigli M, Rigolin GM, Fucili A, Porta MD, Soukhomovskaia O, Malagutti P, Bugli AM, Bragotti LZ, Francolini G, Mauro E, Castoldi G, Ferrari R. CD34+ and endothelial progenitor cells in patients with various degrees of congestive heart failure. *Circulation*. 2004; 110:1209–1212. [PubMed: 15249502]
 37. van den Bosch BJ, van den Burg CM, Schoonderwoerd K, Lindsey PJ, Scholte HR, de Coe RF, van Rooij E, Rockman HA, Doevendans PA, Smeets HJ. Regional absence of mitochondria causing energy depletion in the myocardium of muscle LIM protein knockout mice. *Cardiovascular Research*. 2005; 65:411–418. [PubMed: 15639480]
 38. Vasa M, Fichtlscherer S, Aicher A, Adler K, Urbich C, Martin H, Zeiher AM, Dimmeler S. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circulation Research*. 2001; 89:E1–E7. [PubMed: 11440984]
 39. Wang XXY, Mou Y, Zhao Y, Blankesteyn WM, Hall JL. A role for the beta-catenin/T-cell factor signaling cascade in vascular remodeling. *Circulation Research*. 2002; 90:340–347. [PubMed: 11861424]
 40. Werner N, Kosiol S, Schiegl T, Ahlers P, Walenta K, Link A, Bohm M, Nickenig G. Circulating endothelial progenitor cells and cardiovascular outcomes. *The New England Journal of Medicine*. 2005; 353:999–1007. [PubMed: 16148285]
 41. Wong KBK, Bishop J, Petrou M, Yacoub MH. Clenbuterol induces cardiac hypertrophy with normal functional, morphological and molecular features. *Cardiovascular Research*. 1998; 37:115–122. [PubMed: 9539865]
 42. Wong K, Boheler KR, Petrou M, Yacoub MH. Pharmacological modulation of pressure-overload cardiac hypertrophy: changes in ventricular function, extracellular matrix, and gene expression. *Circulation*. 1997; 96:2239–2246. [PubMed: 9337196]
 43. Wright DE, Cheshier SH, Wagers AJ, Randall TD, Christensen JL, Weissman IL. Cyclophosphamide/ granulocyte colony-stimulating factor causes selective mobilization of bone marrow hematopoietic stem cells into the blood after M phase of the cell cycle. *Blood*. 2001; 97:2278–2285. [PubMed: 11290588]
 44. Yuen AH, Yacoub MH, Smolenski RT. Determination of clenbuterol concentration in human blood using liquid chromatography with electrospray/ion-trap tandem mass spectrometry. *Rapid Commun Mass Spectrom*. 2005; 19:3603–3606. [PubMed: 16276488]

45. Zolk O, Caroni P, Bohm M. Decreased expression of the cardiac LIM domain protein MLP in chronic human heart failure. *Circulation*. 2000; 101:2674–2677. [PubMed: 10851202]

Fig. 1. FACS analysis of bone marrow isolates of MLP^{-/-} saline and MLP^{-/-} clenbuterol treated animals, n=6 in per group. Equal numbers of cells from each animal were stained with mouse IgG anti-CD31-APC. Values are means±standard error. Bars represent percentages as compared to average MLP^{-/-} saline control group

Fig. 2.

Positive correlation between BNP and TIMP-1 mRNA expression in the heart. Expression levels were measured by RT-PCR and normalized to an 18S transcript as an internal control. Triangles denote clen-buterol treated $MLP^{-/-}$ mice (n=5), squares denote saline-treated $MLP^{-/-}$ mice (n=4), and circles denote wild-type mice (n=4). Wild-type mice are in a C57BL6 strain

Fig. 3.
Inverse correlation between circulating LIF-1 levels and ejection fraction. Triangles denote clenbuterol treated $MLP^{-/-}$ mice (n=4), squares denote saline-treated $MLP^{-/-}$ mice (n=3)

Fig. 4.

Effects of clenbuterol on beta 1 and beta 2 adrenergic receptor mRNA levels in the heart of wild-type controls (n=4), MLP $^{-/-}$ treated with saline (n=4), and MLP $^{-/-}$ treated with clenbuterol (n=5). Expression levels were measured by RT-PCR and normalized to an HPRT1 transcript as an internal control. Data are expressed as $\Delta\Delta\text{ct}$. NS not significant. Wild-type mice are in a C57BL6 strain

Fig. 5.

Effect of clenbuterol on beta 1 and beta 2 adrenergic receptor mRNA levels in bone marrow of wild-type controls (n=4), MLP $^{-/-}$ -treated with saline (n=4) and MLP $^{-/-}$ treated with clenbuterol (n=5). Expression levels were measured by RT-PCR and normalized to a cyclophilin A transcript as an internal control. Data are expressed as $\Delta\Delta\text{ct}$ (NS not significant). Wild-type mice are in a C57BL6 strain

Table 1

Transthoracic echocardiographic analysis of MLP^{-/-} mice after 6 weeks of treatment with saline or clenbuterol (*n*=6 each group)

Echocardiographic analysis	MLP ^{-/-} saline	MLP ^{-/-} clenbuterol	<i>p</i> value
Ejection fraction (%)	26.6± 1.06	19.0±0.37	0.032
Body weight (g)	27.7±0.36	28.6±0.25	0.48
LVIDd (mm)	4.68±0.08	4.71 ±0.05	0.92
LVIDs (mm)	3.94±0.08	4.10±0.06	0.612
FS (%)	15.7±0.42	12.2±0.42	0.19
LV mass	171.1 ±4.8	163.6±4.14	0.703

Echocardiograms were analyzed separately by two cardiologists blinded to the study design. Values are means±standard error *LVIDd* left ventricular internal dimensions in diastole, *LVIDs* left ventricular internal dimensions in systole, FS fractional shortening

Table 2

Analysis of circulating factors in the serum of $MLP^{-/-}$ mice treated with either saline (n=3) or clenbuterol (n=4). Values are means±standard error

Circulating factors	$MLP^{-/-}$		
	Saline (n=3)	Clenbuterol (n=4)	p value
Significant			
Apolipoprotein A1 (µg/mL)	91.97±2.76	59± 1.73	0.0001
C-reactive protein (µg/mL)	0.63±0.1	0.34±0.02	0.019
Fibroblast growth factor basic (ng/mL)	0.8±0.04	1.5±0.18	0.019
Leukemia inhibitory factor (pg/mL)	163±2.7	247±15.3	0.0059
Monocyte chemoattractant protein-3 (pg/mL)	74±13.2	116±8.4	0.038
Macrophage-derived chemokine (pg/mL)	293 ±4	470±32	0.0058
Serum glutamic oxaloacetic transaminase (µg/mL)	33.7±3.6	17±2.8	0.0129
Tissue inhibitor of metalloproteinase-type 1 (ng/mL)	0.6±0.1	1.6±0.3	0.044
Not significant			
Epidermal growth factor (pg/mL)	26.2±4.2	67.0±32.1	0.338
Eotaxin (pg/mL)	598±41	1481±359	0.093
Factor VII (ng/mL)	0.9±0.2	2.5± 1.5	0.411
Monocyte chemoattractant protein-1 (pg/mL)	30±1.2	36±4.6	0.317
Monocyte chemoattractant protein-5 (pg/mL)	44 ± 5.1	56±14.2	0.54
Macrophage colony stimulating factor (ng/mL)	2.4±0.1	3.2±0.4	0.182
Tissue factor (ng/mL)	2.2±0.3	6.4±3.7	0.381
Vascular cell adhesion molecule-1 (ng/mL)	1627±158	2138±368	0.313
Vascular endothelial cell growth factor (pg/mL)	133±11	175±17	0.126