Targeted expression of cyclin D2 ameliorates late stage anthracycline cardiotoxicity

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Abstract

Background. Doxorubicin (DOX) is a widely used and effective anti-cancer therapeutic. DOX treatment is associated with both acute and late onset cardiotoxicity, limiting its overall efficacy. Here, the impact of cardiomyocyte cell cycle activation was examined in a juvenile model featuring aspects of acute and late onset DOX cardiotoxicity.

Methods and Results. Two-week old MHC-cycD2 transgenic mice (which express cyclin D2 in postnatal cardiomyocytes and exhibit sustained cardiomyocyte cell cycle activity; D2 mice) and their wild type (WT) littermates received weekly DOX injections for 5 weeks (25 mg/kg cumulative dose). One week after the last DOX treatment (acute stage), cardiac function was suppressed in both groups. Acute DOX cardiotoxicity in D2 and WT mice was associated with similar increases in the levels of cardiomyocyte apoptosis and Ku70 / Ku80 expression (markers of DNA damage and oxidative stress), as well as similar reductions in hypertrophic cardiomyocyte growth. Cardiac dysfunction persisted in WT mice for 13 weeks following the last DOX treatment (late stage), and was accompanied by increased levels of cardiomyocyte apoptosis, Ku expression and myocardial fibrosis. In contrast, D2 mice exhibited a progressive recovery in cardiac function, which was indistinguishable from saline-treated animals by 9 weeks following the last DOX treatment. Improved cardiac function was accompanied by reductions in the levels of late stage cardiomyocyte apoptosis, Ku expression and myocardial fibrosis.

Conclusions. These data suggest that cardiomyocyte cell cycle activity can promote recovery of cardiac function and preserve cardiac structure following DOX treatment.

Key Words: heart failure, cardiomyocyte apoptosis, cardiac regeneration

Introduction

Anthracyclines such as doxorubicin (DOX) are highly effective anti-cancer therapeutics. These agents are also highly cardiotoxic. Acute cardiac responses to anthracycline treatment are transient and usually occur within several days of treatment. Symptoms include hypotension, tachycardia, arrhythmia and reduction of ventricular function.[1-4] Patients can also present with recalcitrant heart failure months or years after anthracycline treatment is terminated, the severity of which is directly related to cumulative drug exposure. It has been estimated that the risk of developing cardiomyopathy is 4% when the dose of DOX is 500–550 mg/m^2, 18% when the dose is 551–600 mg/m^2 and 36% when the dose exceeds 600 mg/m^2.[2, 5] Pediatric patients are particularly sensitive to anthracycline-induced heart failure.[6, 7]

Anthracycline treatment has many adverse consequences for cardiomyocytes. For example, treatment results in a reduction in protein synthesis, inhibition of myogenic transcriptional programs and induction of myofiber degeneration,[reviewed in 8, 9] which collectively are likely to contribute to the transient anthracycline-induced cardiomyocyte atrophy and suppressed cardiac function observed during acute treatment. Treatment also results in increased levels of reactive oxygen species, increased levels of DNA damage and reduced electron transport chain transcriptome activity.[reviewed in 10-13] These changes collectively are likely to contribute to the high rates of cardiomyocyte apoptosis observed following anthracycline treatment *in vitro* and *in vivo*. Although many molecular pathways have been implicated in anthracycline-induced cardiotoxicity, the precise mechanisms which give rise to acute vs. late onset disease remain elusive.

Many forms of cardiac disease are associated with a concomitant loss of cardiomyocytes. This has led to the relatively simple notion that increasing cardiomyocyte renewal rates could stabilize, or even reverse, the progression of heart failure. Accordingly, we have examined the impact of enhanced cardiomyocyte renewal rates in a juvenile model of DOX-induced heart failure which exhibits aspects of acute and late stage cardiotoxicity.[14] We utilized MHC-cycD2 mice (D2 mice), which express cyclin D2 (a key cell cycle regulator) under the control of the cardiomyocyte-specific myosin heavy chain promoter. These animals exhibit sustained increases in postnatal cardiomyocyte S-phase activity, phosphorylated histone H3 expression, and cardiomyocyte division (the latter evidenced by increased cell numbers in dispersed cell preparations) as compared to their wild type (WT) littermates.[15, 16] Interestingly, cardiomyocyte nucleation patterns are normal in these mice.[15] Previous studies have shown that enhanced cardiomyocyte renewal rates in D2 mice are associated with progressive structural and functional recovery following myocardial infarction,[15, 17, 18] cytokine-induced atrial fibrosis,[19] and chronic pressure overload.[16] We show here that the enhanced level of cardiomyocyte renewal in D2 mice is associated with progressive recovery from anthracycline-induced heart failure.

Methods

Please see the Supplemental Data Section for a more detailed description of the methods employed in this study**.**

Mice. This study utilized mice generated by intercrossing MHC-cycD2[15] and MHC-nLAC[20] transgenic animals. The number of mice used to generate each data set are

indicated in the Supplemental Data Figures. All animal protocols were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee.

Echocardiography. Mice were anesthetized with 1.5% isoflurane and two-dimensional images were obtained using a VisualSonics Vevo 770 instrument as described.[21]

Cardiomyocyte DNA synthesis assay. Cardiomyocyte DNA synthesis was monitored via tritiated thymidine incorporation as described.[20, 22]

Statistical analysis. All values are presented as mean ± SEM. Statistical significance was determined using Student's t-test for comparison of two groups, and Two-Way ANOVA with Bonferroni correction for comparison between 3 groups or more. $p<0.05$ was considered significant.

Results

D2 mice exhibit a progressive improvement in cardiac function following DOX-induced cardiotoxicity

To determine the impact of cardiomyocyte cell cycle activity following anthracycline treatment, two week old WT mice and their D2 transgenic littermates were enrolled in a long-term DOX cardiotoxicity study (some of the mice also carried the MHC-nLAC[20] reporter transgene). The mice received 5 weekly injections of saline or 5 weekly injections of DOX (5 mg/kg/injection, 25 mg/kg cumulative dose). Cardiac function was assessed via ultrasound prior to the first injection (i.e., at two weeks of age), one week after the last injection (i.e., 7 weeks of age) and subsequently at 15 and 19 weeks of age. Cardiac function was similar in all groups prior to the initial injection (Figure 1A). Cardiac function remained stable in the saline-treated WT and D2 mice over the course of the study. DOX-treated WT mice exhibited decreased cardiac

function when examined one week after the last treatment (designated the “acute stage”), and cardiac function remained suppressed up to 19 weeks of age (designated the “late stage”), the last time point examined. DOX-treated D2 mice also exhibited decreased cardiac function when examined at the acute stage, albeit with a tendency towards being less severe than what was seen in DOX-treated WT mice. The DOX-treated D2 mice exhibited a progressive restoration of cardiac function, which was normal by 15 weeks of age. Figure 1B shows representative short-axis echocardiograms of saline- and DOX-treated mice (see also Supplemental Tables 1 and 2).

Western blot analyses were performed to confirm expression of the MHC-cycD2 transgene. As expected, mice carrying the transgene exhibited cyclin D2 expression in both the acute and late stages, and expression was not altered by DOX treatment (Figure 2A). Expression of endogenous CDK4 was also elevated in D2 mice as compared to WT mice, in agreement with previous observations.[15] To monitor cardiomyocyte DNA synthesis, the mice received an injection of tritiated thymidine (^{3}H-Thy) four hours prior to sacrifice. After harvest, the hearts were sectioned, and the sections were reacted with the chromogenic β-galactosidase substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL, which is used to identify cardiomyocyte nuclei in mice carrying the MHC-nLAC reporter) and processed for autoradiography. S-phase cardiomyocyte nuclei were readily identified by the presence of silver grains overlaying blue X-GAL reaction product (Figure 2B). In agreement with previous studies,[15, 16, 18] saline-treated D2 mice had markedly elevated levels of cardiomyocyte S-phase activity as compared to saline-treated WT mice (Figure 2C). DOX treatment

resulted in a 5-fold decrease in S-phase activity in the D2 mice when assessed in the acute stage. Surprisingly, reduced levels of cardiomyocyte S-phase activity persisted in late stage D2 mice.

D2 mice exhibit reduced late stage cardiomyocyte apoptosis

Activated caspase-3 immune reactivity was used to quantitate cardiomyocyte apoptosis; since activated caspase-3 is localized in the cytoplasm, early-stage apoptotic cardiomyocytes are identified based on the presence of cytoplasmic activated caspase-3 immune reactivity in rod-shaped cells (Figure 3A).[23] DOX treatment induced cardiomyocyte apoptosis in WT juvenile mice during the acute stage (Figure 3B), and late stage animals exhibited even higher levels of apoptosis despite the termination of treatment (in good agreement with earlier studies).[14, 24] DOX-treated D2 mice exhibited similar levels of acute stage cardiomyocyte apoptosis as was observed for WT mice. In contrast to the WT mice, the level of cardiomyocyte apoptosis did not further increase in late stage DOX-treated D2 mice. Interestingly, the level of cardiomyocyte tritiated thymidine incorporation/mm^2 was greater than the level of cardiomyocyte activated caspase 3 immune reactivity/mm^2 in DOX-treated D2 mice during both the acute and late stages; in contrast, the level of tritiated thymidine incorporation was lower than the level of activated caspase 3 in DOX-treated WT mice (Figure 3C).

Ku (a heterodimer protein complex comprising Ku70 and Ku80) is induced in response to DNA damage,[25] and Ku70 is also thought to function as a transcriptional co-factor in response to oxidative stress.[26, 27] DOX treatment induced Ku expression in WT juvenile mice during the acute stage (Figure 3D), and late stage animals exhibited even higher levels of expression (in good agreement with earlier studies).[24] DOX

treatment induced Ku expression in acute stage D2 mice to a similar degree as was seen in WT mice. Interestingly, Ku levels were reduced in late stage D2 mice.

Myocardial remodeling following DOX treatment

Acute DOX treatment is known to induce cardiomyocyte atrophy in adult hearts[28] and to reduce hypertrophic cardiomyocyte growth in juvenile hearts.[14] Previous studies have shown that minimal fiber diameter (MFD) measurements in histologic sections provide a reliable index of cardiomyocyte hypertrophy or atrophy.[29] MFD was similarly reduced in DOX-treated WT and D2 mice during the acute stage, and was normalized in both groups during the late stage (Figure 4A). Finally, Sirius red/fast green staining was used to quantitate myocardial collagen deposition, which is indicative of fibrosis. No overt difference in myocardial Sirius red content was observed in saline- vs. DOX-treated mice during the acute stage (Figure 4B). DOX-treated WT mice exhibited increased levels of myocardial Sirius red staining during the late stage, indicative of increased fibrosis. In contrast, there was no increase in myocardial Sirius red staining in late stage D2 mice. Figure 4C shows representative images from DOX-treated WT and D2 hearts. Interestingly, cyclin D2 expression had no impact on vascular density (Supplemental Figures 7 and 8).

Discussion

We have previously shown[15, 16] that D2 mice exhibit sustained cyclin D2 expression for up to 12 months of age (the latest time point tested). Transgene exppression is accompanied with cardiomyocyte cell cycle activity, as evidenced by the presence of elevated levels of cardiomyocyte S-phase activity, elevated levels of cardiomyocyte phosphorylated histone H3 immune reactivity, and increased

cardiomyocyte numbers in postnatal hearts. Transgene expression has no detrimental impact on cardiac function in this model. The data presented here demonstrate that the presence of sustained cardiomyocyte cell cycle activity in D2 mice is associated with a progressive recovery in cardiac function in a juvenile model of anthracycline-induced cardiotoxicity. In contrast, cardiac function remained repressed in DOX-treated WT mice. The observed effects in the D2 mice must cell autologous, as the transgene is only expressed in cardiomyocytes (Supplemental Figure 9).[15]

The similar impact on the levels of cardiomyocyte apoptosis, Ku induction and inhibition of developmental hypertrophic growth indicate that cardiomyocytes from WT and D2 DOX-treated mice were subjected to a similar degree of injury at the cellular level during the acute stage. In WT mice, the level of S-phase activity was lower than the level of activated caspase 3 immune reactivity in the acute stage (3-fold) and much lower in the late stage (25-fold), indicating a progressive net loss of cardiomyocytes. The increase in Ku expression observed in the late stage WT mice suggests a model wherein the inability to replace cardiomyocyte loss in response to DOX treatment results in increased myocardial stress, which would in turn contribute to increased levels of cardiomyocyte apoptosis and ultimately myocardial fibrosis.

In contrast, and despite a sustained reduction in tritiated thymidine incorporation, the level of cardiomyocyte S-phase activity (which we have previously shown correlates to renewal in the D2 model, see above) is more than 5-fold greater than the level of cardiomyocyte apoptosis in DOX-treated D2 mice in both the acute and late stages. This suggests that the rate of cardiomyocyte replacement is greater than the drop-out rate (even when one takes into consideration the likely differences in the duration of S-

phase vs. activated caspase-3 immune positivity). The presence of elevated levels of cardiomyocyte cell cycle activity was associated with improved cardiac function and no further induction of cardiac stress (as evidenced by the normalization of Ku expression and the absence of myocardial fibrosis in late stage DOX-treated D2 mice). Thus, the level of cardiomyocyte apoptosis would not be expected to increase in late stage D2 mice (which is what was observed). The presence of similar levels of cardiomyocyte apoptosis in acute and late stage D2 mice (as opposed to the absence of late stage apoptosis) is consistent with a recent study suggesting that DOX induces a permanent change in the mitochondrial gene transcriptome which contributes to sustained cardiomyocyte dysfunction and apoptosis after drug withdrawal.[10]

Although DOX treatment resulted in a similar degree of cardiomyocyte injury in WT and D2 mice at the cellular level, EF was greater in DOX-treated D2 vs. DOX-treated WT mice in the acute stage (although the difference was not statistically different). It should be noted that postnatal D2 mice have a greater cardiomyocyte content than their non-transgenic littermates.[16] Thus, despite a similar impact of DOX treatment at the cellular level, the presence of more cardiomyocytes could readily explain the better cardiac function observed at the organ level in D2 vs. WT mice during the acute stage. In contrast, the progressive restoration of normal cardiac function in late stage DOX-treated D2 mice likely results from increased levels of cardiomyocyte renewal and a concomitant decrease in adverse remodeling.

While a reduction in cardiomyocyte S-phase is to be expected in DOX-treated D2 mice during the acute stage due to the anti-proliferative activity of anthracyclines, it is not clear why S-phase levels remain repressed during the late stage, particularly as

DOX treatment had no impact on expression of the transgene or endogenous CDK4. It is possible that the pool of proliferating cardiomyocytes in D2 mice (that is, the fraction of cyclin D2-expressing cardiomyocytes able to enter the cell cycle) is rather small. If so, 5 successive rounds of DOX injection would be expected to markedly deplete the pool, resulting in a permanent reduction in the thymidine labeling index. Alternatively, it is possible that there is a labile cardiomyogenic stem cell population which is progressively eliminated by DOX treatment over time. However, recent results using a lineage trace system demonstrated that DOX treatment actually enhances cardiomyogenic activity in putative c-kit progenitor cells,[30] thus a permanent reduction in transgene-induced cardiomyocyte S-phase activity would not be anticipated (particularly since the MHC-cycD2 transgene would not be expected to be expressed in a progenitor cell which has not yet adopted a cardiac phenotype).

In summary, the data here support the notion that increased rates of cardiomyocyte renewal can lead to normalization of cardiac function in a juvenile model of DOX-induced cardiotoxicity. Such an approach might be viable to ameliorate, or reverse, late onset cardiomyopathy sometimes encountered in cancer patients treated with anthracyclines.

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Conflicts of Interest

None declared.

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Figure Legends

Figure 1. Cardiac function in saline- and DOX-treated WT and D2 mice. (A) Fractional Shortening (FS) in saline- and DOX-treated WT and D2 mice at 2, 7, 15 and 19 weeks of age. Vertical arrows indicate the age at saline or DOX injection. "*": $p < 0.05$ vs. WT-Saline; "†": $p<0.05$ vs. D2-Saline; "‡": $p < 0.001$ vs. D2-DOX; Two-Way ANOVA with Bonferroni correction). See Supplemental Figure 1 for a scatter plot of the FS values for the individual animals studied. (B) Representative short axis echocardiograms from saline- and DOX-treated WT and D2 mice during the acute and late stages.

Figure 2. Transgene expression and cardiomyocyte DNA synthesis in acute stage and late stage mice. (A) Western blot analysis of cyclin D2 and CDK4 expression in saline- and DOX-treated WT and D2 mice during the acute and late stages. (B) Image of a section from a D2 mouse heart harvested 4 hours after ^{3}H-Thy injection (the mouse also carried the MHC-nLAC reporter transgene). The section was reacted with X-GAL to identify cardiomyocyte nuclei and processed for autoradiography to identify ^{3}H-Thy incorporation. The presence of black silver grains over blue X-GAL reaction product is indicative of an S-phase cardiomyocyte nucleus (see arrows; bar = 10 microns). (C) Quantitation of cardiomyocyte ^{3}H-Thy labeling in saline- and DOX-treated WT and D2 mice during the acute and late stages (Two-Way ANOVA with Bonferroni correction). See Supplemental Figure 2 for a scatter plot of the ^{3}H-Thy labeling indices for the individual animals studied.

Figure 3. Cardiomyocyte apoptosis and myocardial stress in acute and late stage mice. (A) Representative image of an activated caspase-3 immune reactive cardiomyocyte from a DOX-treated mouse. Bar = 25 microns. (B) Quantitation of activated caspase-3

immune reactive cardiomyocytes ($CSP3^+$ CM) per mm^2 in hearts from saline- and DOX-treated WT and D2 mice during the acute and late stages (Two-Way ANOVA with Bonferroni correction). See Supplemental Figure 3 for a scatter plot of the cardiomyocyte activated caspase-3 values for the individual animals studied. (C) Levels of tritiated thymidine-positive cardiomyocytes and activated caspase-3-positive cardiomyocytes per mm^2 myocardial tissue from DOX-treated WT and D2 mice during the acute and late stages (Two-Way ANOVA with Bonferroni correction). (D) Western blot analysis of Ku70 and Ku80 expression in hearts from saline- and DOX-treated WT and D2 mice during the acute and late stages. See Supplemental Figure 4 for quantitation of the Ku70 and Ku80 signals.

Figure 4. Myocardial atrophy and fibrosis in acute and late stage mice. (A) Quantitation of cardiomyocyte MFD in hearts from saline- and DOX-treated WT and D2 mice during the acute and late stages. See Supplemental Figure 5 for a scatter plot of the average MFDs for the individual animals studied. (B) Quantitation of Sirius red myocardial content in hearts from saline- and DOX-treated WT and D2 mice during the acute and late stages (Two-Way ANOVA with Bonferroni correction). See Supplemental Figure 6 for a scatter plot of the myocardial Sirius red content for the individual animals studied. (C) Representative images of Sirius red/fast green-stained heart sections prepared from saline- and DOX-treated late stage WT and D2 mice (bar = 50 microns).

Figure 1.

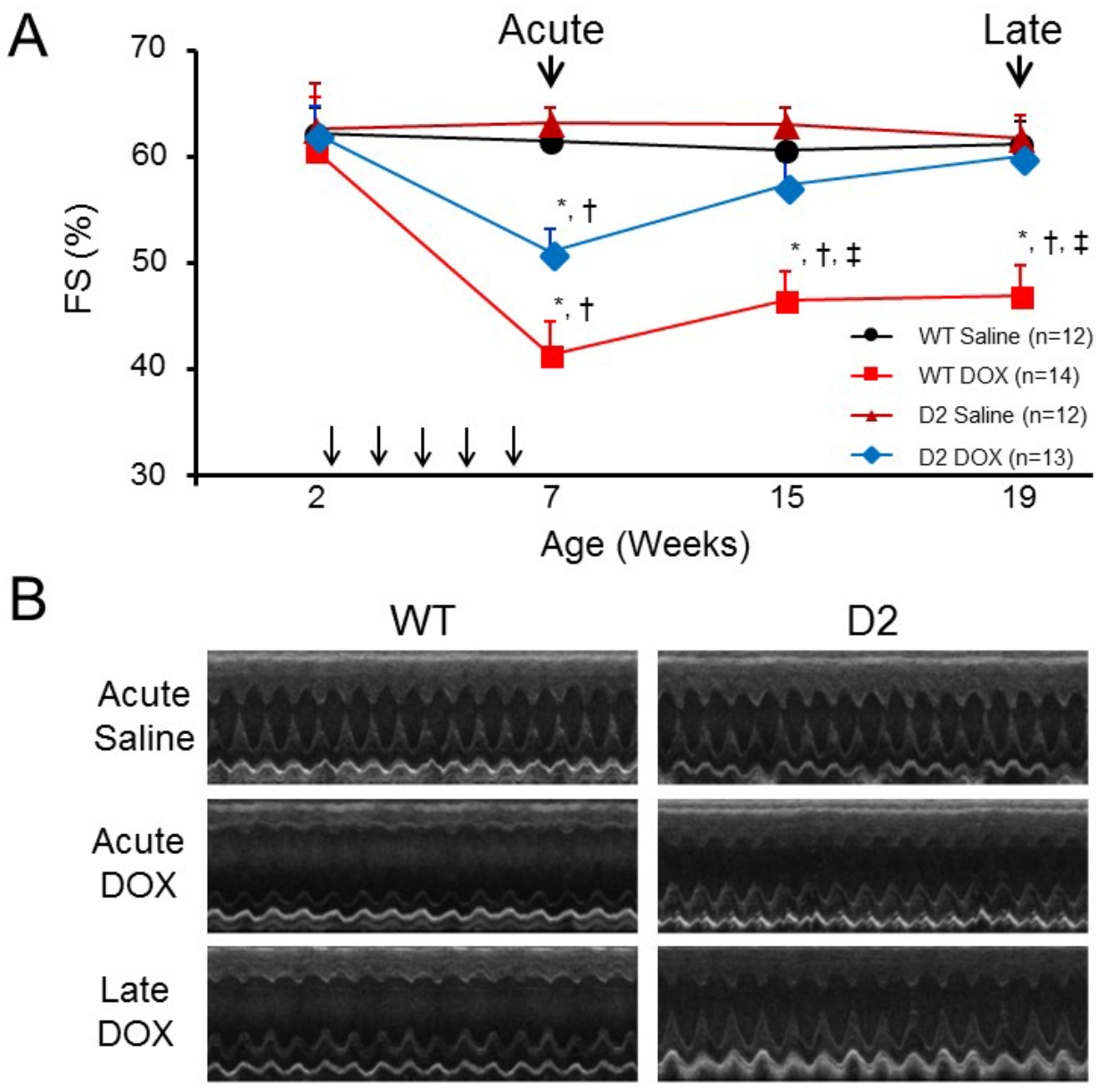
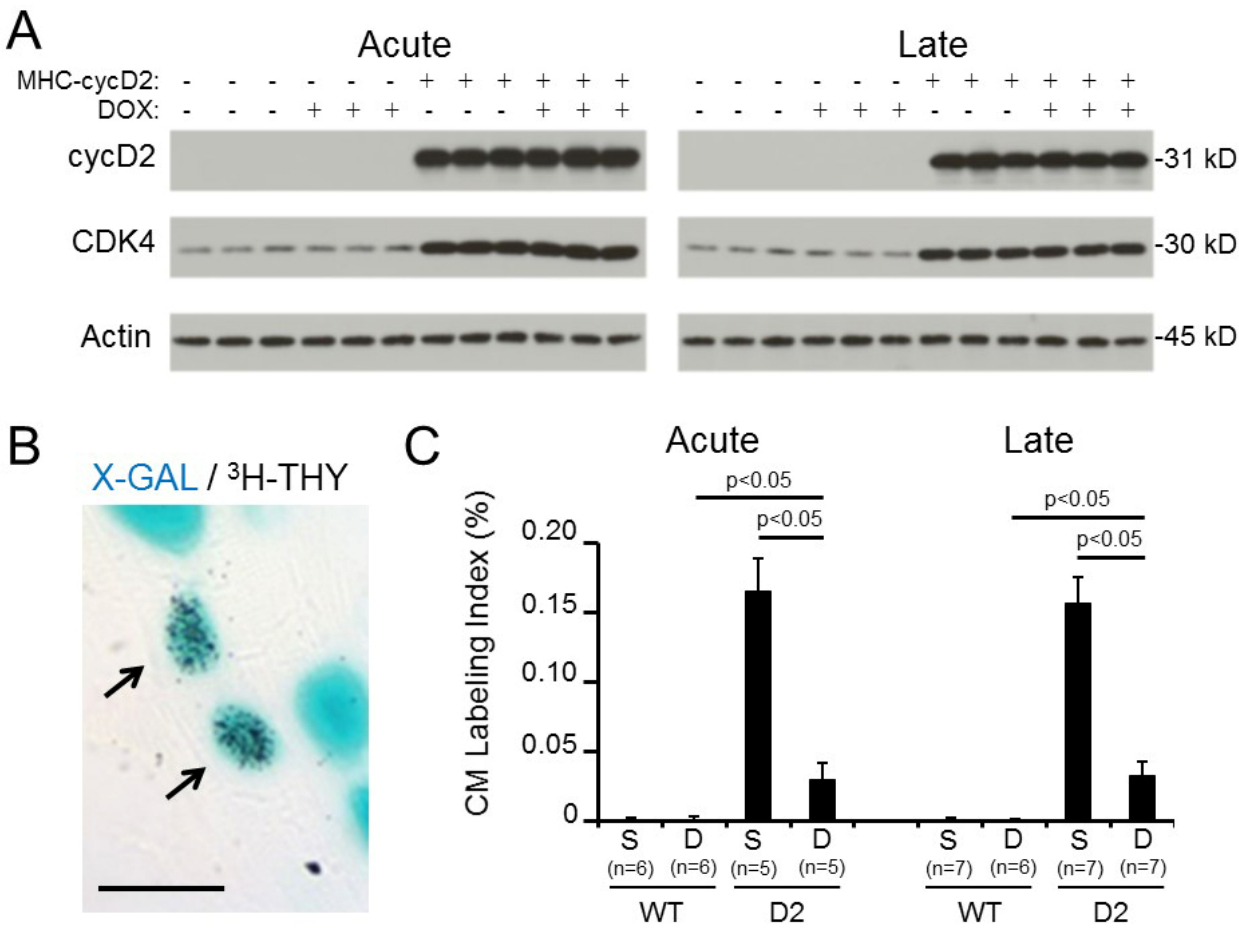


Figure 2.



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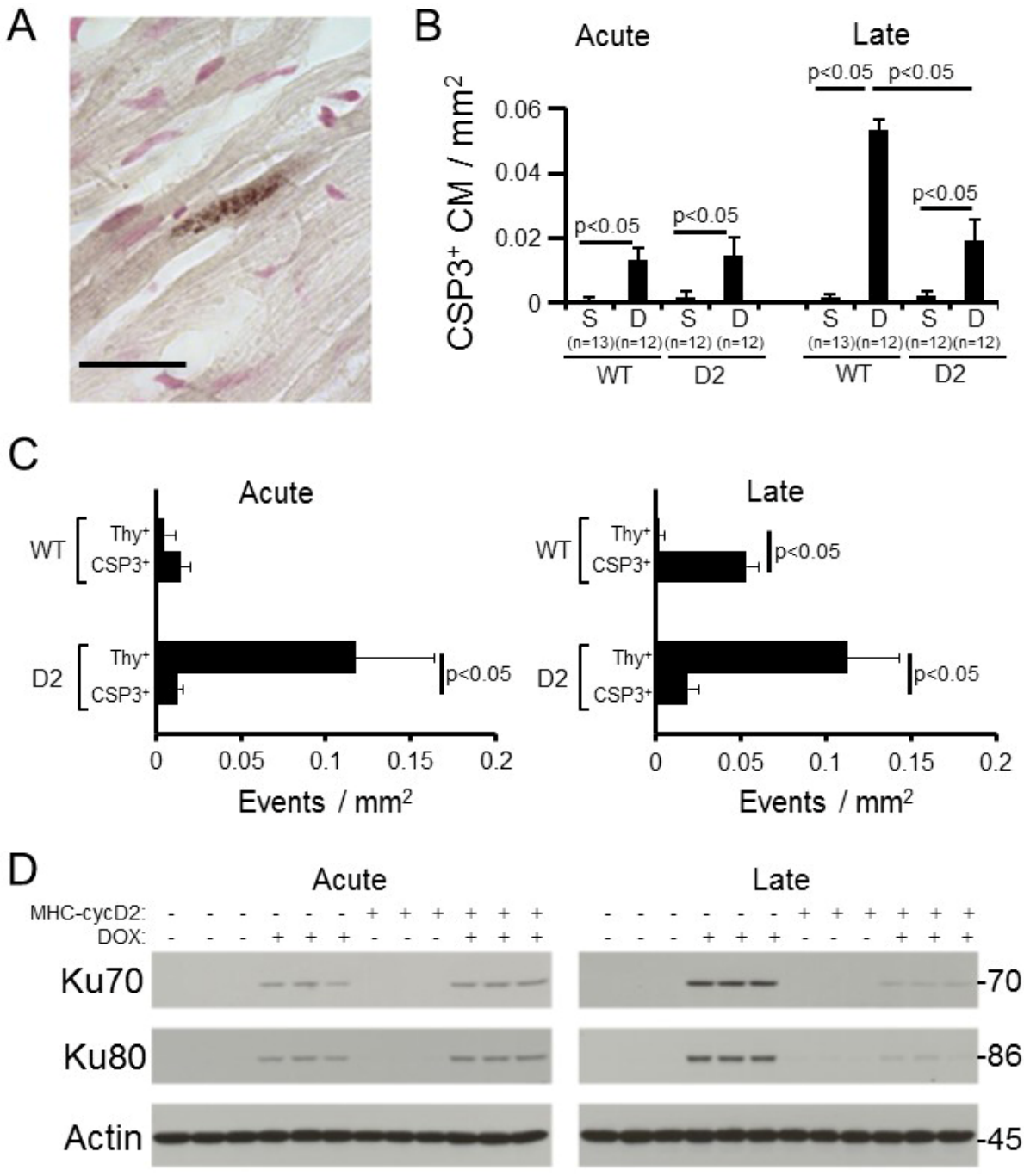

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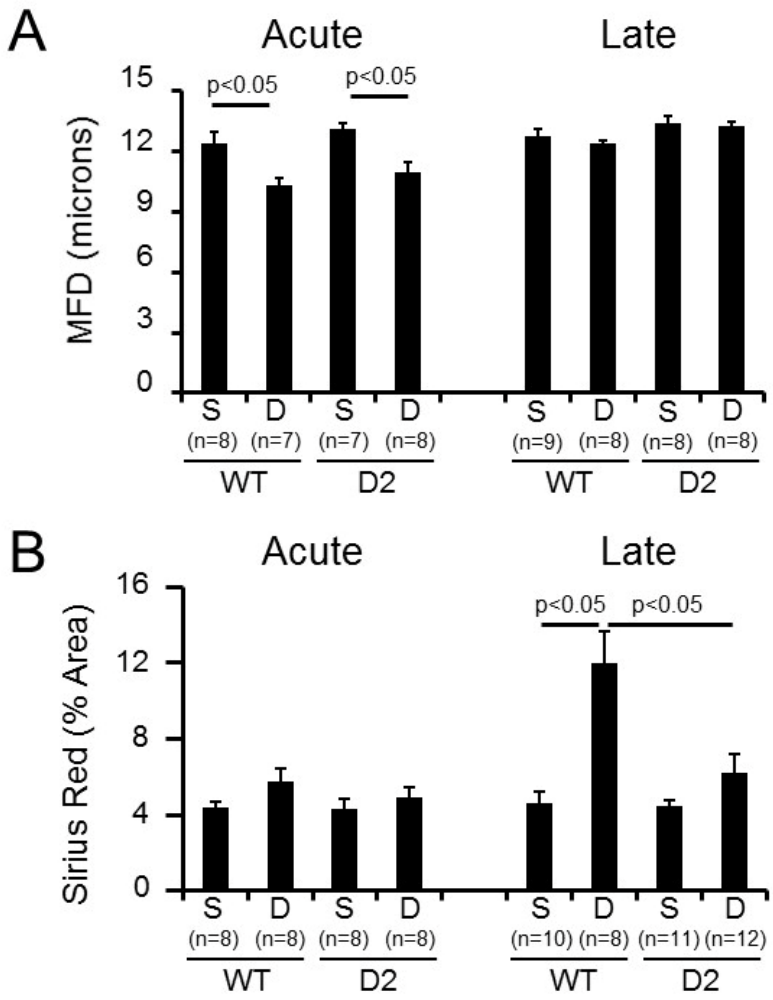

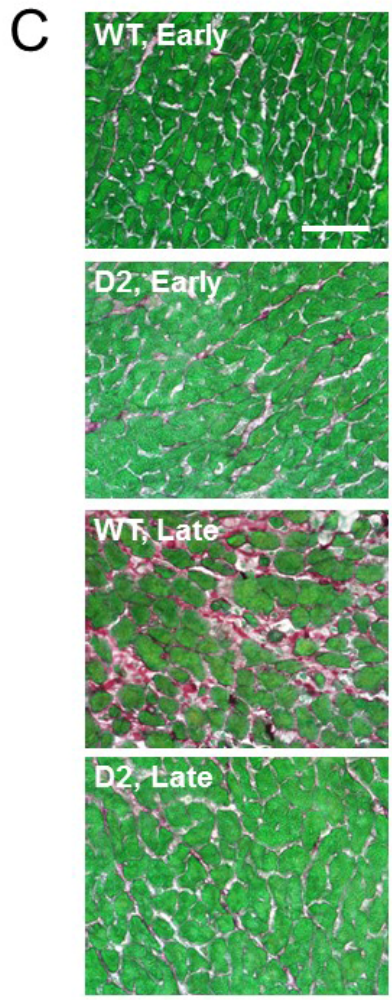

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