Limitations of the Pax7-creER\textsuperscript{T2} transgene for driving deletion of \textit{Nf1} in adult mouse muscle

MATTHEW A. SUMMERS\textsuperscript{1,2}, KATHY MIKULEC\textsuperscript{1}, LAUREN PEACOCK\textsuperscript{1}, DAVID G. LITTLE\textsuperscript{1,2} and AARON SCHINDELER*\textsuperscript{1,2}

\textsuperscript{1}Orthopaedic Research & Biotechnology, The Children’s Hospital at Westmead, Westmead and \textsuperscript{2}Discipline of Paediatrics & Child Heath, Faculty of Medicine, University of Sydney, Camperdown, NSW, Australia

ABSTRACT Neurofibromatosis Type 1 (NF1) is an autosomal dominant genetic disorder that results in a variety of characteristic manifestations. Prior studies have shown reduced muscle size and global skeletal muscle weakness in children with NF1. This associated weakness can lead to significant challenges impacting on quality of life. Pre-clinical studies using a muscle-specific NF1 knockout mouse have linked this weakness to an underlying primary metabolic deficiency in the muscle. However, the neonatal lethality of this strain prevents analysis of the role of NF1 in adult muscle. In this study, we present the characterization of an inducible muscle-specific NF1 knockout strain (\textit{Nf1Pax7i\textsuperscript{f/f}}) produced by cross breeding the Pax7-\textit{CreER}\textsuperscript{T2} strain with the conditional \textit{Nf1flox/flox} line. Tamoxifen dosing of 8-week old \textit{Nf1Pax7i\textsuperscript{f/f}} mice led to recombination of the floxed allele in muscle, as detected by PCR. Detailed phenotypic analysis of treated adult mice over 8 weeks revealed no changes in bodyweight or muscle weight, no histological signs of myopathy, and no functional evidence of distress or impairment. Subsequent analysis using the \textit{Ai9 Cre-dependent tdTomato} reporter strain was used to analyse labelling in embryos and in adult mice. Cell tracking studies identified a lower than expected rate of integration of recombined satellite cells into adult muscle. In contrast, a high persistent contribution of embryonic cells that were \textit{Pax7+} were found in adult muscle. These findings indicate important caveats with the use of the Pax7-\textit{CreER}\textsuperscript{T2} strain and highlight a need to develop new tools for investigating the function of \textit{NF1} in mature muscle.

KEY WORDS: neurofibromatosis type 1 (NF1), muscle weakness, myopathy, Cre-ER\textsuperscript{T2}, cre/loxP

Introduction

Neurofibromatosis type 1 (NF1) is an autosomal dominant genetic disorder with a global incidence of 1:3000 (Lammert et al., 2005). Individuals with NF1 can present with multiple manifestations that have a significant clinical impact, including tumor development (Ratner and Miller, 2015, Ferner and Gutmann, 2013), learning difficulties (Lehtonen et al., 2013, Levine et al., 2006), and musculoskeletal abnormalities (Patel and Stacy, 2012, Summers et al., 2015). In recent years, clinical reports of muscle weakness have spurred research into the role of \textit{NF1} in muscle. While early reports of motor deficits in NF1 children were attributed to nerve dysfunction (Feldmann et al., 2003), reports of reduced muscle size (Stevenson et al., 2005), impaired exercise capacity (Johnson et al., 2012, de Souza et al., 2013), and muscle weakness (Cornett et al., 2015, Johnson et al., 2012, Stevenson et al., 2012, Souza et al., 2009) suggested primary muscle dysfunction.

The \textit{NF1} protein product neurofibromin has a key role in lineage specification and cell differentiation, and is classically known as a Ras GTPase-activating protein (Ratner and Miller, 2015, DeClue et al., 1991). Neurofibromin expression has been shown to be up regulated during myoblast differentiation \textit{in vitro} (Gutmann et al., 1994) and \textit{in vivo} (Kossler et al., 2011), and recent studies have implicated \textit{Nf1} in the regulation of muscle development and lipid metabolism (Summers et al., 2015). Notably, double-inactivation of \textit{Nf1} in muscle using the \textit{MyoD-Cre} transgene resulted in a severe developmental phenotype, including neonatal lethality and muscle lipid accumulation (Sullivan et al., 2014). Notably, the lipid deposition in \textit{Nf1\textsubscript{myoD+}} muscle was not associated with changes in...
metabolic enzyme activity or altered expression of lipid transporters. However, in adult muscle from a limb-specific \( \text{Nf1} \) knockout mouse (Sullivan et al., 2014), double-inactivation of \( \text{Nf1} \) resulted in substantial alterations in mitochondrial metabolic enzymes levels (Sullivan et al., 2014). These data suggest either developmental age-related compensatory differences, or unrelated genetic differences between mouse strains may be confounding interpretations. Thus, further mouse model development in the field is needed.

A limitation with the aforementioned studies is that \( \text{Nf1}_{\text{MyoD}^-} \) mice feature developmental loss of the \( \text{Nf1} \) gene. During development, muscle progenitors are migrating and fusing to form the early myotubes, and loss of \( \text{Nf1} \) may impede this process. This may poorly reflect the role of \( \text{NF1} \) in the maintenance of mature muscle. Thus, it was hypothesized that an \( \text{Nf1} \) knockout mouse targeted for post-mitotic muscle would be able to (1) survive post weaning and (2) allow us to investigate the role of \( \text{NF1} \) in the maintenance of mature muscle solely in a postnatal context, and would mechanistically contrast with prior developmental models.

The development of tamoxifen inducible CreER\(^{T2}\) systems represents a significant advance in the control of DNA recombination. This is achieved by fusion of Cre to an estrogen receptor (ER) ligand binding domain. The Cre-ER fusion protein is sequestered to the cell cytosol via interactions with chaperone proteins, such as heat-shock protein 90 (HSP90). Upon interaction with an estrogen analogue, such as 4-OH-tamoxifen (Tam), this Cre-ER fusion interaction is disrupted, allowing Cre recombinase to translocate to the nucleus and bind its target \( \text{loxP} \) sites (McLellan et al., 2017). Tam-induced Cre recombination was first demonstrated in cells in 1995 (Metzger et al., 1995). A later iteration (termed Cre-ER\(^{T2}\)) has modified ER domains resulting in a 10-fold greater sensitivity to Tam (Indra et al., 1999). In the field of muscle disease, inducible Cre-ER\(^{T2}\) systems are particularly useful when studying developmental and/or post-mitotic gene function. Accordingly, several myogenic Cre-ER\(^{T2}\) drivers have recently been developed and made available to the muscle research community; including the Pax3-Cre-ER\(^{T2}\) (Southard et al., 2014), Pax7-Cre-ER\(^{T2}\) (Lepper and Fan, 2012) and MyoD-Cre-ER\(^{T2}\)strains (Southard et al., 2014).

Since characterization of the \( \text{Pax7}^+ \) cell pool as muscle stem cells, numerous studies have unequivocally demonstrated their requirement for muscle regeneration following injury (Sambasivan et al., 2011, Murphy et al., 2011, McCarthy et al., 2011, Lepper et al., 2011, Relaix and Zammit, 2012). In the absence of injury however, it was assumed that this cell population was quiescent in adult muscle (Montarras et al., 2013). However, recent cell tracking studies have confirmed a progressive and pervasive contribution of satellite cell nuclei into uninjured adult myofibers (Keefe et al., 2015a, Pawlikowski et al., 2015a). For example, in 8-week-old mice, pulse labelling of \( \text{Pax7}^+ \) cells followed by a 14-day chase found

![Fig. 1. Tamoxifen dosing of adult \( \text{Nf1}_{\text{Pax7}^-} \) mice results in limited recombination in muscle. (A)](image)

Genomic DNA PCR finds detectable levels of flox site recombination following TAM dosing (lane 2), compared with non-dosed animals (lane 1) quadriceps muscle extracts. Lane 3 shows robust detection of recombined floxed allele from the constitutive Cre expressing MyoD-Cre\(Nf1^f/f\) strain as a positive control. No significant differences seen in bodyweight from baseline, or muscle weights when compared to controls, either (B,C) 1 week following Tam dosing \( (n=3) \), (D,E) 4 weeks following dosing \( (n=3) \), or (F,G) 8 weeks following dosing \( (n=3) \). * tamoxifen dosing days. \( \text{TA} \), Tibialis Anterior; \( \text{EDL} \), extensor digitorum longus; \( \text{Sol} \), soleus; \( \text{Gas} \), gastrocnemius; \( \text{Quads} \), quadriceps.
50–60% of hind limb myofibers labelled positive (Pawlikowski et al., 2015a). As late as 6–12 months of age, sedentary mice were shown to maintain integration of new satellite cell nuclei in up to 30% of limb myofibers (Keefe et al., 2015b). This unique property of continuing integration makes the Pax7+ cell an ideal vehicle for the genetic manipulation of the muscle syncytium.

Herein we aimed to inactivate Nf1 in satellite cells by employing Cre-ERT² technology and conditional lineage control using the muscle-specific Pax7 promoter. We hypothesized that Nf1 inactivation in Pax7+ cells will result in a progressive muscle phenotype in adult mice, as Cre-expressing satellite cell nuclei are progressively integrated into established myofibers. To test this, we crossed the Pax7-CreERT² line with the Nf1flox/fox line to generate tamoxifen-inducible muscle-specific Nf1 knockout mice. Nf1Pax7i f/f mice were dosed once daily for 5 days from 8 weeks of age, with 1.5mg/20g bodyweight tamoxifen, a dosing regimen that is previously published to elicit Cre-mediated recombination rates exceeding 90% in adult animals (Pawlikowski et al., 2015a). The primary study outcome measures included changes to mouse bodyweight and muscle weight over time, and muscle histopathology following tamoxifen injections. Follow up experiments were then done using the Ai9 red fluorescent reporter strain (Madisen et al., 2010) to validate the sensitivity and lineage specificity of the Pax7-Cre driver.

Results

To test our primary hypothesis that Nf1 inactivation in adult Pax7+ cells would result in a progressive myopathy, Nf1Pax7i f/f mice were dosed with tamoxifen from 8 weeks of age. Mice were monitored daily for body weight and sub-groups culled at three time points: 1 week (n=3), 4 weeks (n=3), and 8 weeks (n=3) following tamoxifen injection. Major hind limb muscles were harvested and weighed prior to storage and analysis.

Genomic DNA PCR for the recombinated floxed allele at 8 weeks confirmed a level of tamoxifen induced Cre-mediated recombination in muscle (Fig. 1A). As a positive control, tissue from Nf1MyoD-/- mice was tested and showed an equivalent band representing the recombined allele, albeit at a greater intensity. Contrary to our hypothesis, no changes in bodyweight or muscle weight were seen at 1 week (Fig. 1 B-C), 4 weeks (Fig. 1 D-E), or 8 weeks (Fig. 1 F-G) following tamoxifen injections. Neither absolute muscle weight nor muscle weight normalised to bodyweight (Fig. 1) showed significant differences. Furthermore, Oil Red O staining with a hematoxylin counterstain showed no evidence of myopathic changes, including fibrosis, centralized nuclei, altered fibre size, or lipid droplet accumulations 8 weeks following injections (Fig. 2).

Fig. 2. Histological analysis of major hind limb muscles finds no evidence of myopathy. (A) Oil Red O staining of mid-belly muscle cross sections from tamoxifen dosed animals reveals no evidence of myopathic changes, or lipid droplet accumulations 8 weeks following injections. (B) Positive control for Oil Red O staining showing lipid droplet accumulation in Nf1-/- muscle from Myod-Cre Nf1flox/flox stain. TA, Tibialis Anterior; EDL, extensor digitorum longus; Sol, soleus; Gas, gastrocnemius; Quads, quadriceps. Scale bars: (A) 100 μm, (B) 50 μm.
In the absence of evidence for a progressive myopathy, the efficiency of Pax7-lineage cell incorporation in the muscle was examined. Thus the Pax7-Cre-ER\textsuperscript{T2} line was crossed with the Ai\textsuperscript{9} Cre-responsive TdTomato (TdTom) red fluorescent reporter mouse (Madisen et al., 2010). Following prenatal and postnatal tamoxifen dosing, embryonic and adult cell tracking studies were performed.

For developmental studies, pregnant females bearing Pax7-Cre-ER\textsuperscript{T2}-Ai\textsuperscript{9} positive pups were dosed with 0.75\,mg/20\,g bodyweight tamoxifen, at two different embryonic time points: ED9.5 & 15.5. Pups were born and weaned. Mice grew normally post-weaning and were culled at 8 weeks of age for analysis. When Pax7+ cells were labelled developmentally at ED15.5, analysis of muscle cross-sections showed robust and homogenous TdTom labelling of muscle myofibers (Fig. 3A); consistent with a high rate of nuclei integration during the late embryonic and postnatal period. Furthermore, myofiber labelling was tamoxifen-dosing dependent (Fig. 3C) and muscle specific, as no reporter expression was seen in other cell types, such as adjacent bone cells (Fig. 3B). A mosaic pattern of muscle fibre fluorescence was observed when Pax7+ cells were labelled at ED9.5 (Fig. 3D), suggesting a proportion of the early myogenic progenitors do not yet express Pax7 at this time point.

For adult labelling studies, Pax7-Cre-ER\textsuperscript{T2}-Ai\textsuperscript{9} positive mice were dosed with tamoxifen from 8 weeks of age. Adult mice were culled for analysis 4 weeks following injections, and fibre labelling was assessed by fluorescent histology of hind limb muscle tissues. In contrast to prior studies, tamoxifen dosing in adult mice resulted in limited labelling of satellite cells at the muscle fibre periphery, along with labelling of only a small number of whole muscle fibres 4 weeks following injections (Fig. 3F). Though fibre labelling was substantially less than predicted, labelling remained tamoxifen-dependent, as no signal could be detected in non-dosed control muscle (Fig. 3E).

**Discussion**

Our data raise several questions about factors affecting inducible gene targeting in muscle. PCR analysis from genomic DNA extracted from tamoxifen-treated mice could detect Cre-mediated recombination, and no leakiness was seen in the absence of tamoxifen treatment. However, while the band intensities of induced mice samples were less than that of muscle-specific knockout mice, intensities did not differ substantially, and without a quantitative PCR assay direct comparisons cannot be made.

Ultimately, the lack of phenotypic response in adult Nf1\textsuperscript{Pax7}i\textsubscript{r} mice to tamoxifen treatment was attributed to poor recombination within mature muscle fibres. This could be the result of either insufficient satellite cell integration or alternatively a downregulation of Cre-expression by myonuclei following satellite cell fusion. These data poorly align with other reported uses of the Pax7-CreERT\textsubscript{2} line in the context of adult muscle. Indeed, the capacity of Pax7-lineage cells to contribute to new muscle fibres may not be associated with their capacity to drive the continued expression of transgenes in adult muscle fibres. Studies have shown Pax7 expression is substantially down-regulated following terminal differentiation and myofiber integration (Olguin et al., 2007). To achieve widespread recombination throughout the muscle syncytium using Pax7-Cre drivers, satellite cell nuclei must maintain some Cre-recombinase expression following myofiber fusion. In this study we hypothesized that sufficient Cre-recombinase protein would be present in the cytosol of fusing satellite cells to affect the existent myonuclei in a muscle fibre. Moreover, the nuclei from Pax7-lineage satellite cells would definitively show Cre exposure. However, our data indicate that this is not the case for the Pax7-CreERT\textsubscript{2} system.

Published studies showing the continued integration of Pax7+ cells into adult muscle are largely limited to qualitative assessments of fluorescent labelling (Pawlikowski et al., 2015b, Keefe et al., 2015a). Our adult cell tracking data show that we achieved substantially less than 50% labelling of established fibres from 8-weeks of age. These data contrast with studies showing upwards of 60% of myofibers labelled by this time point (Pawlikowski et al., 2015a). Furthermore, personal communications with the group of Dr Brya Mathews and A/Prof Ivo Kalajzic using same reporter strain (Matthews et al., 2016), confirmed our findings show a substantially lower than expected rate of satellite cell integration from this time point. Alternatively, it is possible that limited tissue availability of active tamoxifen, and/or a reduced recombination

**Fig. 3.** Fluorescent cell tracking of Pax7+ satellite cells finds lower than expected rates of integration into adult muscle. (A) TdTom reporter expression in adult quadriceps muscle when Pax7+ cells are labelled at ED15.5. Expression is muscle specific and non-leaky (B) * bone cells adjacent to labelled muscle that are not TdTom positive. (C) No reporter expression detected in muscle from animals that did not receive tamoxifen. (D) Labelling Pax7+ cells at ED9.5 resulted in a mosaic labelling pattern of adult muscle fibers. Tamoxifen dosing of 8-week-old adults resulted in limited satellite cell labelling (F) at the fiber periphery (examples indicated by arrow head), and (E) labelling of only a limited number whole muscle fibers (indicated by arrows) compared to non-dosed controls. Scale bars: 200 \( \mu \text{m} \).
efficiency in adult mice may explain our results. This has been a subject of recent conjecture, and methods for optimal satellite cell targeting and the need for quantifying recombination efficiencies has been discussed in light of differing results from Pax7-Cre-ER\textsuperscript{T2} studies (Brack, 2014).

Perhaps the most intriguing finding from our validation studies using the Pax7CreER\textsuperscript{T2}-Ai9 fluorescent reporter strain is that when Pax7+ cells were labelled early in development, at ED9.5, we saw a mosaic expression pattern in adult muscle fibers (Fig. 3D). These data suggest that a portion of early committed myogenetic progenitor cells are not Pax7+, but by ED15.5 all express Pax7 (Fig. 3A-B). Indeed, many studies support this conclusion. It has been shown that the developmental expression of Pax3 precedes Pax7, and plays a non-redundant role in myogenesis (Kassar-Duchossoy et al., 2005, Lepper and Fan, 2010). For example, global Pax7+ mice survive until weaning and show no signs of muscle malformation (Mansouri et al., 1996). In contrast, global Pax3 inactivation is lethal, and embryos fail to develop body or limb skeletal muscles (Tajbakhsh et al., 1997). Later studies examining expression timing found that the dermomyotomal cells that parent myogenic cells of the limbs are heavily Pax3+ at ED9.75, however do not strongly express Pax7 until ED11.5 (Kassar-Duchossoy et al., 2005), and are not completely muscle restricted until ED12.5 (Lepper and Fan, 2010). Understanding these embryonic dynamics will significantly aid future studies targeting the Pax7+ cell pool.

For future studies, methodologies able to dissect the mechanisms of prenatal versus postnatal Pax7+ cell fusion would be valuable. One key comparison would be assessing a quantitative measure of myonuclear expression of Cre-recombinase with a measure of myonuclear recombination. One potential technique to achieve this would be a customized fluorescent in situ hybridization (FISH) protocol. This protocol would also be valuable in the context of analysing patient NF1 muscle biopsies. It has been long questioned whether the muscle weakness seen in individuals with NF1 is caused by loss of the second NF1 allele. Double inactivation of NF1 has been observed in other manifestations including tumours (Serra et al., 1997), and in bone lesions found in tibial pseudarthrosis patients (Stevenson et al., 2006). Alternatively, a qPCR technique may be optimised to give an accurate ratio of floxed (unrecombined) and recombined alleles.

Conclusion

Successful gene targeting using the Cre-ER\textsuperscript{T2} system requires several factors working optimally. Notably, tissue-wide penetration of tamoxifen, and complete Cre activation and expression in the target cell population. Our data further highlight the need for thorough examination of persistent Cre-expression and recombination efficiencies when using cre/loxP systems. Furthermore, efficient myofiber gene targeting using the vehicle of the satellite cell remains to be explored.

It will be important for future studies modelling NF1-muscle weakness to consider the caveats of developmental versus post-natal double inactivation in mice, particularly as double inactivation in human NF1 muscle is yet to be determined. The currently available NF1 muscle-specific and limb-specific double knockout mouse models have thus far proven insightful, and will likely continue to be a valuable resource in the field.

Materials and Methods

Mouse genetics and breeding

Animal experiments were approved by the Westmead Hospital Animal Ethics Committee or the Children’s Hospital at Westmead/Children’s Medical Research Institute Animal Ethics Committee. For fluorescent reporter studies Pax7-Cre-ER\textsuperscript{T2} transgenic mice and Ai9-tdTom red fluorescent reporter mice (sourced from Jackson laboratory USA) were crossed to produce first generation experimental heterozygous animals Pax7-Cre+/- Ai9+/- . For Pax7+ cell Nf1 knockout experiments Pax7-Cre-ER\textsuperscript{T2} transgenic mice were first crossed with Nf1floxFlox-/+ mice to produce first generation heterozygous animals Pax7-Cre+/- Nf1floxFlox-/+ . They were then backcrossed with the parental Nf1floxFlox+/- strain to generate experimental homozygous animals Pax7-Cre-ER\textsuperscript{T2}- Nf1floxFlox-/+ . Samples were collected at weaning for genotyping by quantitative real-time PCR for the Cre and Nf1flox alleles (Transnet YX, U.S.A). All colonies were maintained on a C57/B6 background.

Tamoxifen dosing

1g Tamoxifen powder (Sigma-Aldrich) was first dissolved in 2ml 100% ethanol and 1ml corn oil (Sigma-Aldrich) using sonication and heated as needed. The solution was then made up to a 100ml stock (10mg/ml concentration) using corn oil, and stored in aliquots at -20°C protected from light. A working solution was prepared from the stock solution by diluting to required concentration with corn oil. Intrapelletional injections were performed using a 26-gauge needle.

PCR assay for flox allele recombination

Forward primers (P1) CTCCTAGCTGATGTTGTACCTGA, and reverse primers (P2) CATCTGCTGCTCTTAGAGGAACA, were used to detect the recombined floxed sequence in genomic DNA extracted from quadriceps muscle, using the following program: 1x 95°C 3min, 40 cycles (95°C 30sec, 50°C 30sec, 72°C 1min), 72°C 7min. PCR products were run on a 1% agarose gel.

Muscle histology

Muscle tissue were harvested from animals at cull, weighed, then surface coated in Tissue-Tek® O.C.T. Compound (SAKURA FINETEK USA), placed on a thin piece of tin foil and frozen in isopentane (2-methyl butane) supercooled in liquid nitrogen, and stored at -80 °C. 8um sections were cut on a Leica CM1950 Clinical Cryostat, and captured on Superfrost™ Plus Microscope Slides (Fisher Scientific, USA) and stored at 4°C prior to lipid staining.

Fluorescent microscopy

Muscle sections were fixed in 4% PFA for 2min, and then rinsed in deionized water (dH\textsubscript{2}O) for 30 sec. Cell nuclei were stain with a 1:10,000 4',6-diamidino-2-phenylindole (DAPI):dH\textsubscript{2}O solution for 1min. Rinsed in dH\textsubscript{2}O for 30 sec again before cover slipping using Aquatex® aqueous mounting agent. tdTom and DAPI fluorescent signal was captured using an Olympus BX61 fluorescent microscope.

Oil Red O Staining

Oil Red O (ORO) stock solution was prepared by dissolving 0.5g ORO powder (Sigma-Aldrich) in 100ml isopropanol (100%), on a plate shaker at 31°C overnight. A working solution was made by diluting stock solution 3:2 in dH\textsubscript{2}O and filtered through a 75um cap filter.

Muscle sections were fixed in 4% PFA for 2min, and then rinsed in dH\textsubscript{2}O for 30 seconds. Slides were dipped 4 times in 60% isopropanol: dH\textsubscript{2}O solution before incubating in ORO stain solution for 30min. Slides were again rinsed in 60% isopropanol: dH\textsubscript{2}O, counter stained in hematoxylin for 2min, then cover slipped using Aquatex® aqueous mounting agent.
Acknowledgments
This study was supported by a grant from the Children’s Tumor Foundation (20138-05-009).

References

Further Related Reading, published previously in the Int. J. Dev. Biol.

DYRK2 displays muscle fiber type specific function during zebrafish early somitogenesis
Wei Sun, Shuang Jiao, Xungang Tan, Peijun Zhang and Feng You
https://doi.org/10.1387/ijdb.160175sj

FoxD1 protein interacts with Wnt and BMP signaling to differentially pattern mesoderm and neural tissue
Hanna Polevoy, Anastasia Malyarova, Yuri Fonar, Sara Elias and Dale Frank
https://doi.org/10.1387/ijdb.160300df

Building functional units of movement-generation and movement-sensation in the embryo
Peleg Hasson, Talila Volk and Adi Salzberg
https://doi.org/10.1387/ijdb.160279as

Models of amphibian myogenesis - the case of Bombina variegata
Leokadia Kiełbówna and Marta Migocka-Patrzalek
https://doi.org/10.1387/ijdb.160370mm

mRNA cycles through hypoxia-induced stress granules in live Drosophila embryonic muscles
https://doi.org/10.1387/ijdb.103172al

Tumor suppressor genes as negative growth regulators in development and differentiation
D H Gutmann
http://www.intjdevbiol.com/web/paper/8901192